JOURNAL OF THE NATIONAL CANCER INSTITUTE

National Institutes of Health • Public Health Service

Volume 24, Number 1

January 1960

THE National Cancer Institute is privileged to honor the memory of Jesse P. Greenstein with this Memorial Issue of invited papers from a few of his many friends and colleagues. It is altogether fitting and proper that this be done because of the many extraordinary contributions Dr. Greenstein made to science and to the Institute during the twenty years he was a member of the Staff, the last thirteen of which were spent as Chief of the Laboratory of Biochemistry. We hope this Memorial Issue will serve as an indication of the high esteem we all felt for him.

CARL G. BAKER, M.D.
Special Editor for this Issue

Jesse Philip Greenstein

Late Chief

Laboratory of Biochemistry

National Cancer Institute

Jesse Philip Greenstein

1902–1959

CANCER RESEARCH lost one of its giants when, on February 12, 1959, death came suddenly to Dr. Jesse P. Greenstein. His many contributions enriched the field of biochemistry and were in the forefront in bringing order to cancer biochemistry, and his extraordinarily productive career continued undiminished until his death. During the past two decades, his Laboratory at the National Cancer Institute was an outstanding center of research on cancer, on amino acids and peptides, and on nutrition. His comparative studies on enzyme activities of cancer and normal tissues and the masterful synthesis published as his monograph, *Biochemistry of Cancer*, in 1947 constituted a high light in cancer research. His investigations on amino acids, peptides, and nucleoproteins have enhanced our knowledge of biochemistry, enzymology, organic and physical chemistry. His extensive work on the resolution of amino acids has provided optically active forms of these substances on a large scale, which is leading to vastly increased use of these formerly rare materials. The indefatigable energy and selfless devotion with which he pursued his own scientific researches, and the high quality and permanent value of his results, have earned him the lasting respect and admiration of all who knew him.

Probably the most characteristic traits of Jess Greenstein were his intense dedication to his chosen field and his deep respect for the individual. His mind was a massive vault filled with scientific knowledge, yet while he was humble when he viewed the living miracles of the world about him, he possessed a never-ending curiosity to unlock the mystery of their elusively concealed secrets. To his contemporaries he was a living dynamo, primed with a source of energy that permitted him to work at a vigorous pace for sixteen to eighteen hours a day, seven days a week, the only waste product of which seemed to be the smoke which emerged from an apparently endless chain of cigars. This boundless energy and his insistence on excellence, his high moral courage, and his great knowledge of and insight into science, coupled with a firm yet understanding and jovial nature, served as a continual source of inspiration to his younger scientific associates whose eager compulsion to follow his lead from example often led them to scientific achievements of which they had believed themselves incapable. Despite the prodigious output of work, he was friendly, warm, and good humored. His door was always open to scientific associates who sought his counsel and advice, and those who worked closely with him remember the many pleasant conversations often held in the early morning hours, conducted while he was performing organic syntheses—inducing a new crystallization by vigorously scratching with a stirring rod the wall of an Erlenmeyer flask containing a thick syrup, and with a reac-

tion mixture or two refluxing in the background. At scientific meetings and other events away from the Laboratory he was a sparkling companion. On several occasions when outstanding scientists visited Bethesda, he arranged informal gatherings at his home, which afforded his colleagues a most delightful opportunity to know these prominent investigators in an atmosphere of warm conviviality.

He was modest and unassuming about his own accomplishments, but displayed an overt pride in the achievements of his younger associates in whom he took a sincere personal interest, on whom he would freely lavish praise, and to whom he served as a leader in the finest sense. Because of his personal characteristics and his extraordinary scientific accomplishments he attracted to his Laboratory a stream of capable young scientists—from England, France, Japan, Germany, Canada, Israel, China, Korea, Greece, Italy, Belgium, Switzerland, Brazil, and India—eager to follow his lead. He gave a warm and generous welcome to the visitors who frequented his Laboratory in a never-ending stream.

For his outstanding contributions Dr. Greenstein received many honors. He was appointed, in 1949, as a member of the American delegation to a Cancer Colloquium called by Pope Pius XII at the Vatican in Rome, received the Carl Neuberg Medal in 1950, the Distinguished Service Award of the Department of Health, Education, and Welfare in 1954, and the Hillebrand Award of the American Chemical Society in 1958. He was, in 1956, a visiting lecturer at Japanese universities, during which time he was appointed to honorary memberships in the Japanese Biochemical Society and the Japanese Foundation for Cancer Research. He had served, in 1955, as Chairman of the Division of Biological Chemistry of the American Chemical Society and, at the time of his death, was an editor of the *Archives of Biochemistry and Biophysics*, co-editor with Professor A. Haddow of *Advances in Cancer Research*, on the Committee of Scientific Advisors, Institute of Microbiology, Rutgers University, and a member of the Committee on Biochemistry of the National Research Council, holding the chairmanship of the Subcommittee on Amino Acids.

Jesse Greenstein was born in 1902 in New York City, where he attended the city public schools. From 1920 to 1922 he was engaged as a technician in the Bureau of Foods and Drugs of the City of New York, a position which whetted his appetite for a scientific career. By 1926 he had earned his Bachelor of Science degree, *cum laude*, from the Polytechnic Institute of Brooklyn, where he acted as managing editor of the school newspaper and where his extraordinary physical stamina served him in good stead as a member of the wrestling team; he received a signal honor from this same Institute exactly thirty years later with the award of a Certificate of Achievement as one of its most distinguished graduates. During 1926–27 he was employed in New York with Seil, Putt and Rusby, Inc., a commercial laboratory providing analytical and consultation services on foods and drugs, where he gained valuable experience in obtaining definitive results by the most expedient means and in the shortest possible time.

In 1927 Jesse Greenstein entered Brown University where, under the guidance of Professor C. A. Kraus, he earned his Master of Science degree in 1928

and fulfilled the requirements for his Doctor of Philosophy degree in biochemistry two years later with a dissertation on the effect of various electrolytes on the dissociation constants of amino acids and simple peptides. This initial excursion into scientific research was distinguished by the fact that it was accomplished when pure amino acids were still chemical rarities and when even glycine, the most readily accessible of the amino acids, was generally procured from acid hydrolysates of hippuric acid which, in turn, had been isolated from the urine of mammals fed sodium benzoate. An opportunity to continue and extend these studies to the ionization of complex polypeptides was afforded during 1930–31, when Dr. Greenstein served as a National Research Council Fellow at Harvard under Professor Edwin J. Cohn; about half of this period was devoted to the isolation of the trivalent amino acids (lysine, histidine, etc.) required for these studies from protein hydrolysates, then their only source. These years at Harvard were succeeded by a year's work at the Kaiser Wilhelm Institute in Dresden under Professors Max Bergmann and Leonidas Zervas, who had just developed the revolutionary carbobenzoxy method for the synthesis of peptides. With the aid of these gifted scientists, Dr. Greenstein successfully applied the carbobenzoxy method to the synthesis of lysylglutamic acid and lysylhistidine, peptides that could not have been procured by the means hitherto available. More important, it was this association more than any other that was to have a profound and lasting influence on the pattern of his future researches, for it was here that he both developed a mastery of organic chemical techniques that was extraordinary among the biochemists of his generation and gained an intimate knowledge and deep respect for the Emil Fischer School of Chemistry whose high standards he continually strived to maintain. Jesse Greenstein had little reason to suspect that in only a few years he would have occasion to pay periodic calls at the Rockefeller Institute to renew his warm friendship with Professor Bergmann, who was forced to flee from a Nazi-dominated Germany, or that nearly twenty-five years later he would have occasion to engage again in fruitful collaboration in his own Laboratory with Professor Zervas, who in the meantime had returned to his homeland in Greece.

On his return to the United States in 1932, Dr. Greenstein spent one year as a research associate under Professor C. L. A. Schmidt at the University of California at Berkeley. With him he carried a collection of peptides that was unique for the 1930's, and here he resumed his earlier studies on the influence of chemical structure on ionizable groups. In 1933 he returned to Harvard where until 1939 he was actively and simultaneously engaged as a tutor in the biochemical sciences, as a research associate in Professor E. J. Cohn's Laboratory at Harvard Medical School, and as an instructor for Professor L. J. Henderson's course in biological chemistry. He was an inspiring and effective teacher, both in the classroom and during individual conferences, to the large number of students under his charge. Despite the arduous nature of these duties, he was astonishingly productive in research. He continued to synthesize new polyvalent amino acids and peptides, correlated their ionization constants with their structure, and determined their solubility, dielectric constants, electrostriction, and other physical constants. In addition, he discovered that guanidinium and methylguanidinium ions are highly effec-

tive agents for the denaturation of proteins and that porphyrindin is an excellent reagent for determining by oxidative titration the sulfhydryl groups liberated during protein denaturation. He achieved the first synthesis of cysteinylcysteine and its oxidation to the cyclic peptide, cystinylcystine; nearly twenty years later, he was to resume these studies in an attempt to identify more clearly the products arising from oxidation of the optically active forms of cysteinylcysteine.

With the opening of the National Cancer Institute in 1939, Dr. Greenstein accepted a position as a biochemist under Dr. Carl Voegtlin, the Institute's first Director, and thus entered what was to become the most prominent phase of a remarkable career. An exceptionally able group was assembled at Bethesda by Dr. Voegtlin, and the Institute was, in Dr. Greenstein's own words ". . . what appears to have been something of a unique experiment in the organization of cancer research. Several independent groups of professional investigators trained in various disciplines, such as pathology, genetics, physics, and chemistry were brought together under one roof and encouraged to undertake voluntarily, on a mutually independent basis, cooperative research on problems which interested them, and which could only be solved by effort from several directions. . . . [Dr. Greenstein went on to] speak for the stimulation which such types of cooperation afford, and for the cordiality and mutual helpfulness which cooperation on a voluntary and independent basis is capable of eliciting and sustaining among professional investigators of both different and similar disciplines." In this atmosphere, with a wealth of experimental tumors available, he embarked on an extensive study of biochemistry of cancer with the extraordinary energy and vitality he had displayed formerly. In addition to the pioneer studies on nucleoproteins, both from normal and neoplastic tissues, he systematically investigated the enzyme activities of a variety of tumors and of their normal tissue counterparts. Publications resulting from this work constituted a major portion of the early volumes of this *Journal*, and the resulting extensive data permitted him to make several general statements about transplanted tumors: (*a*) Each normal tissue is characterized by an individual pattern of enzymatic activity that serves to distinguish it from all other tissues, and tumors qualitatively possess the same enzymes as normal tissues; (*b*) the enzymatic pattern of a tumor is largely independent of its age, of its growth rate, and of the strain of animal in which it is grown; (*c*) when a normal tissue becomes neoplastic, many of the functional activities markedly decrease or are lost altogether, and the range of enzymatic values for the tumors, which usually lies between the extremes for normal tissues, is much narrower among tumors than among normal tissues; (*d*) tumors tend to converge, enzymatically, to a common type of tissue. These general conclusions have been reinforced by subsequent work and have been extended to other tumor components, such as vitamin and amino acid levels. Dr. Greenstein also studied the biochemical effects of tumors on the animal hosts bearing them. In general, he found that tumors produce essentially similar systemic effects in the hosts, irrespective of tumor type. One example extensively studied by Dr. Greenstein was the reduction in liver-catalase activity in animals bearing rapidly growing tumors at some distant site. He clearly demonstrated the direct

effect of tumors on the liver catalase by showing that the catalase activity rapidly returned to normal levels upon removal of the tumors. On the basis of his findings Dr. Greenstein postulated that this effect on the liver catalase might be produced by a toxic substance produced in the tumor and carried by the blood to the liver. His pioneering studies in this area have been a focal point for investigations of numerous workers throughout the world.

A critical summary of these investigations was included in his grand synthesis of the then current knowledge in the field: *Biochemistry of Cancer* (published in 1947 and extensively revised in 1954). The importance of this work was aptly summarized by a most distinguished scientific contemporary who stated that apart from Dr. Greenstein's other scientific contributions, "his contributions to cancer research alone would have more than sufficed to establish him among the leading biochemists of his time." The monograph continues to inform, instruct, and inspire cancer investigators and will continue to do so for years to come. Its widespread influence is evident from the fact that it is one of the most frequently cited references in the cancer literature.

The tremendous impact of Dr. Greenstein's research activities played a decisive role in the growing importance and prestige of the National Institutes of Health where, in 1946, he was appointed as Chief of the newly created Laboratory of Biochemistry of the National Cancer Institute. As Chief of the Laboratory he exhibited great wisdom, and his decisions were made with an aim toward excellence in the Laboratory always in the forefront. His valued counsel was frequently sought in matters relating to the operations at Bethesda. He was proud to be a scientist in the Federal Government and always insisted that the scientists' viewpoints not be lost among administrative rulings. Heavy administrative burdens, added to the daily heightening pressures of a continually expanding research program, seemed only to serve as a stimulus to greater effort and, indeed, initiated what was probably the most productive phase of his career. During the year of his appointment as Laboratory Chief and the subsequent one, over forty scientific papers and the splendid monograph were published over Dr. Greenstein's name. He brought to his staff a group of young researchers, most of whom were originally trained in medicine, and he and his associates launched a notable series of studies on dehydropeptides and dehydropeptidases, on intracellular peptidases, on the enzymatic desulfuration of cystine peptides and the enzymatic deamidation of glutamine and asparagine, on the deamination, dephosphorylation, and depolymerization of nucleic acids, and on a host of other problems.

It was during this period that Dr. Greenstein became increasingly impressed both with the desirability of employing optically pure peptide substrates in the study of the powerful peptidases of normal and neoplastic tissues, as well as with the need for optically active amino acids from which these substrates could be prepared. He was troubled by the fact that this latter need could then be met only by employing commercial L-amino acid preparations, which were more often than not scarce, expensive, and of extremely poor quality, or by resorting to the tedious and time-consuming process of either isolating the desired material from a protein hydrolysate or resolving the pertinent

racemate through the use of an alkaloid. As a result of this concern, it was natural that when in the course of their studies on the hydrolysis of acylated amino acids he and his colleagues observed that only the L-form of the amino acid derivative was hydrolyzed, it was immediately recognized as a route to the preparation of optically active amino acids. He embarked on a search for a general amino acid resolution procedure which culminated in what was probably his most outstanding contribution to amino acid chemistry. This search was successfully concluded with the isolation from hog kidney of the two separate enzymatic preparations, designated as renal acylase I and renal amidase, which were capable of hydrolyzing asymmetrically only the L-isomers of N-acylated DL-amino acids and DL-amino acid amides, respectively, and hence permitted the separation and preparation of the L- and D-isomer of a given amino acid in high yield and with an optical purity that was greater than 99.9 percent. The power of this general enzymatic resolution approach is evidenced by the fact that it was applied by Dr. Greenstein and his associates, within the space of a few short years, to the preparation of the L- and D-isomers of some 60 different amino acids, a larger variety of amino acids than has been resolved by all other resolution procedures combined. It is applicable on a micro- or macro-scale, the latter permitting the production of between one and two kilograms of both antipodes per day. Its use, both commercially and in private laboratories, has made available a large variety of L- and D-amino acids in greater quantity, of a higher purity, and at a significantly lower cost than was previously possible. The importance of these optically pure compounds in research is attested by the virtually staggering number of requests for samples received from scores of investigators throughout the world—requests which were honored by Dr. Greenstein in his characteristically generous fashion.

The resolution studies were essentially complete by 1955, though the procedure was still to yield additional fruits in the three areas of research to which Dr. Greenstein then turned his efforts: (1) amino acid chemistry; (2) peptide chemistry; and (3) nutritional and metabolism studies in the whole organism. In extensive and comprehensive investigations on a series of complex diasymmetric amino acids, a masterful study was made of their synthesis, resolution, structural configuration, physical characterization, and enzymatic behavior. From this area of activity, he derived some of his greatest pleasures and intellectual stimulation.

In continuing his work on peptide chemistry, Dr. Greenstein greatly extended his earlier work on the oxidation products of cysteinylcysteine. He and his younger associates, together with his old friend Professor Zervas, whom he invited as a guest worker to his Laboratory in 1955 and again in 1957, developed a new and convenient method for the synthesis of the highly complex N-terminal and C-terminal arginine peptides, effected the first unequivocal synthesis of arginylarginine, studied disulfide interchange in peptides of cystine, and prepared the first pure unsymmetrical linear peptide of cystine.

The ready availability of both optical isomers of amino acids allowed Dr. Greenstein and his associates to initiate nutritional and metabolic studies in the intact organism not previously possible. Studies were conducted on the

toxicity of amino acids, individually and in mixtures, in some 10,000 rats, and the function of arginine in overcoming the toxic effects of ammonia was established, a finding which is being further evaluated in hepatitis and cirrhosis patients. An extensive series of investigations involving a completely water-soluble, chemically defined diet was undertaken in rats, and normal lifespan, reproduction, and lactation were demonstrated for these animals through several generations. Initial clinical work indicates the usefulness of these special diets in conditions requiring parenteral nutrition, particularly in clinical metabolic studies, and the complete chemical definition of the diet permits unique experiments in intermediary metabolism in the intact subject.

Of his scientific findings, Dr. Greenstein imparted freely, in a style of writing distinguished by its elegance, conciseness, and clarity. His command of the English language was superb, an attribute which allowed him to express himself in a manner exactly suited to the occasion. Some of his administrative memoranda commending those under his supervision were masterpieces of concinnity, singular in government communications. In his nearly 300 scientific articles with more than sixty colleagues he was always generous in assuring that recognition was given to his young associates. His last major writing effort, begun in 1955 with one of us (M. W.) and essentially complete at the time of his death, was a comprehensive treatise on the *Chemistry of the Amino Acids*, a definitive three-volume work of some 3,000 pages embracing the physical, organic, biochemical, and analytical aspects of amino acid and peptide chemistry. It is scheduled to appear in 1960.

Although Dr. Greenstein did not indulge in luxury, he enjoyed good food, reveled in the delights of blueberry pie, and smoked a ceaseless chain of cigars, interrupted only occasionally by a pipe. His interests were broad and diverse, and he read extensively in the areas of theology, history, philosophy, and literature. Indeed, often he would return home from his Laboratory about two or three o'clock in the morning, read until nearly dawn, catch a few hours of sleep, and then, apparently fully refreshed, would eagerly embark on another full day of scientific labor. Yet despite the supreme pleasure and satisfaction he attained from his scientific pursuits, he felt a deep sense of regret from the fact that his vigorous and rigorous professional activity caused him to lose many hours which might otherwise have been spent with his understanding and devoted wife and his beloved children. It was primarily for the reason that he and his family might spend more moments together that he became a boating enthusiast during the last three years of his life and purchased a 22-foot cabin cruiser; and it was typical of Jesse Greenstein to engage enthusiastically and intensively even in this newly acquired hobby, for he took and passed with honors several courses in marine navigation, received several certificates from the Washington Area Power Squadron, and was, at the time of his death, pursuing a course in advanced navigation and one in marine engine maintenance.

During the early morning hours of February 10, 1959, Dr. Greenstein was suddenly afflicted with a massive cerebral hemorrhage which culminated in coma almost immediately and in death two days later. His loss came as a great shock to all who knew him. He is survived by his widow Lucy Mitchell Greenstein, his son Michael, his daughter Mrs. Louise Brill, his sister Mrs.

Naomi G. Shor, and his brother Dr. Nathan Greenstein. He is also survived by numerous younger scientists in whom he imbued his zeal, his enthusiasm for science, his high standards, and his appreciation of the value of hard work—and this too is one of his great contributions. The hiatus created by the death of an individual such as Jesse P. Greenstein can never be adequately filled, and for this we are much the poorer, but for the very reason that he was with us, we have been left a good deal richer and perhaps a little wiser.

It was not to be expected that this same individual, who had been responsible for so many incredible and brilliant achievements during his lifetime, was yet to be responsible for a remarkable and dramatic event on the very day of his own funeral. Such an event had its origin some two weeks earlier, when Dr. Greenstein, unbeknown to relatives, friends, or most intimate associates, commissioned an art dealer in Bethesda to transcribe for him a passage from the Bible onto a parchment, and to mount the latter in a frame suitable for hanging on the wall of his beloved Laboratory. On the very morning prior to his unexpected and fatal affliction, he selected a place to hang his as yet undelivered transcription and requested one of the Laboratory technicians to affix a support in the glazed concrete block wall over his desk, just below the pictures of Professors C. A. Kraus, E. J. Cohn, M. Bergmann, and L. Zervas, the four people he felt most responsible for shaping the course of his scientific career. On the very morning of his funeral, the transcribed and framed Biblical passage was delivered to his Laboratory, where it was seen for the first time by his scientific associates and where it presently hangs in the same place he had chosen for it on his Laboratory wall. It reads:

> Whatsoever thy hand findeth to do, do it
> with thy might, for there is no work, nor device, nor
> knowledge, nor wisdom in the
> grave, whither thou goest.
>
> Ecclesiastes 9: 10

Milton Winitz
Herbert A. Sober
Vincent E. Price
Robert E. Greenfield
Sanford M. Birnbaum
Carl G. Baker

Bibliography

1930–1934

Electrometric determinations of the dissociation of glycocoll and simple peptides. *J. Gen. Physiol. 14: 255, 1930.* (With P. H. Mitchell.)

Studies of the peptides of trivalent amino acids. I. Titration constants of histidyl-histidine and of aspartyl-aspartic acid. *J. Biol. Chem. 93: 479, 1931.*

Studies of the peptides of trivalent amino acids. II. Titration constants of tyrosyl-tyrosine and of glycyl-tyrosine. *J. Biol. Chem. 95: 465, 1932.*

The titration constants of α,β-diaminopropionic acid and their relation to the constants of various isomers. *J. Biol. Chem. 96: 499, 1932.*

Synthese von Peptiden des *d*-Lysine: Lysyl-*d*-glutaminsäure und *d*-Lysyl-*l*-histidin. *Ber, deutsch. chem. Ges. 65: 1692, 1932.* (With M. Bergmann and L. Zervas.)

Studies of the peptides of trivalent amino acids. III. The apparent dissociation constants, free energy changes, and heats of ionization of peptides involving arginine, histidine, lysine, tyrosine and aspartic and glutamic acids and the behavior of lysine peptides toward nitrous acid. *J. Biol. Chem. 101: 603, 1933.*

1935–1939

Studies of multivalent amino acids and peptides. I. The synthesis of certain tetravalent amino acids and their derivatives. *J. Biol. Chem. 109: 529, 1935.*

Studies of multivalent amino acids and peptides. II. The synthesis of certain derivatives of lysyl-glutamic acid. *J. Biol. Chem. 109: 541, 1935.*

Studies of multivalent amino acids and peptides. III. The dielectric constants and electrostriction of the solvent in solutions of tetrapoles. *J. Am. Chem. Soc. 57: 637, 1935.* (With J. Wyman, Jr., and E. J. Cohn.)

The solvent action of neutral salts upon peptides in solutions of low dielectric constant. *Proc. Am. Soc. Biol. Chemists, 1935.* (With E. J. Cohn and T. L. McMeekin.)

Studies of multivalent amino acids and peptides. IV. The apparent dissociation constants of α-aminotricarballylic acid and of glycyl-α-aminotricarballylic acid. *J. Biol. Chem. 110: 619, 1935.* (With N. R. Joseph.)

Studies of multivalent amino acids and peptides. V. Cystine cyamidene. *J. Biol. Chem. 112: 35, 1935.*

Studies of multivalent amino acids and peptides. VI. The action of proteolytic enzymes on certain synthetic substrates. *J. Biol. Chem. 112: 517, 1936.*

Further studies of dielectric constant and electrostriction of amino acids and peptides. *J. Am. Chem. Soc. 58: 463, 1936.* (With J. Wyman, Jr.)

On a relation between the dissociation constants of substituted aliphatic acids and the distance between the dissociating and the substituted groups. *J. Am. Chem. Soc. 58: 1314, 1936.*

The synthesis of isocitric acid from citric acid. *Proc. Am. Soc. Biol. Chemists, 1936.*

The effect of certain substituted dithio acids on the blood sugar of rabbits. *Proc. Am. Soc. Biol. Chemists, 1936.* (With H. B. Friedgood.)

Studies in the physical chemistry of amino acids, peptides and related substances. VIII. The relation between the activity coefficients of peptides and their dipole moments. *J. Am. Chem. Soc. 58: 2365, 1936.* (With E. J. Cohn, T. L. McMeekin, and J. H. Weare.)

Studies of multivalent amino acids and peptides. VII. Derivatives of *dl*-α-aminotricarballylic acid. *J. Biol. Chem. 116: 463, 1936.*

Studies of multivalent amino acids and peptides. VIII. The synthesis of bisanhydro-*l*-cystinyl-*l*-cystine and other diketo-*l*-piperazines of cystine. *J. Biol. Chem. 118: 321, 1937.*

The synthesis of crystalline *l*-cysteinyl-*l*-cysteine hydrochloride. *Proc. Am. Soc. Biol. Chemists, 1937.*

Studies of multivalent amino acids and peptides. IX. The synthesis of *l*-cystinyl-*l*-cystine. *J. Biol. Chem. 121: 9, 1937.*

A synthesis of homoarginine. *J. Org. Chem. 2: 1, 1937.*

Peptides, peptidases and diketopiperazines. *In* Chemistry of the Amino Acids and Proteins (Schmidt, C. L. A., ed.). *Springfield, Ill., Charles C Thomas, Chapt. 6, 1938.*

Studies of multivalent amino acids and peptides. X. Cystinyl peptides as substrates for aminopolypeptidase and dipeptidase. *J. Biol. Chem. 124: 255, 1938.*

The polarity of aminocyclohexane carboxylic acids. *Proc. Am. Soc. Biol. Chemists, 1938.* (With J. Wyman, Jr.)

Alicyclic amino acids. *J. Am. Chem. Soc. 60: 2341, 1938.*

Sulfhydryl groups in proteins. I. Egg albumin in solutions of urea, guanidine, and their derivatives. *J. Biol. Chem. 125: 501, 1938.*

Studies on the physical chemistry of cystinyl peptides. *J. Biol. Chem. 125: 515, 1938.* (With F. W. Klemperer and J. Wyman, Jr.)

Sulfhydryl groups in proteins. II. Edestin, excelsin, and globin in solutions of guanidine hydrochloride, urea, and their derivatives. *J. Biol. Chem. 128: 233, 1939.*

Studies of multivalent amino acids and peptides. XI. The synthesis of diglycyl-*l*-cystine. *J. Biol. Chem. 128: 241, 1939.*

Aspartylhistidine. *J. Biol. Chem. 128: 245, 1939.* (With F. W. Klemperer.)

Denaturation of myosin. *J. Am. Chem. Soc. 61: 1613, 1939.* (With J. T. Edsall and J. W. Mehl.)

Book review: Fortschritte der Biochemie by F. Haurowitz (Steinkopff, T., ed.). Dresden-Blasewitz, Germany, 1938. In *J. Am. Chem. Soc. 61: 2211, 1939.*

Further studies on the physical chemistry of cystine peptides. *J. Biol. Chem. 129: 681, 1939.* (With F. W. Klemperer and J. Wyman, Jr.)

Sulfhydryl groups in proteins. III. The effect on egg albumin of various salts of guanidine. *J. Biol. Chem. 130: 519, 1939.*

1940–1944

The effect of denaturing agents on myosin. I. Sulfhydryl groups as estimated by porphyrindin titration. *J. Biol. Chem. 133: 397, 1940.* (With J. T. Edsall.)

Sulfur groups in proteins and the problem of denaturation. *Nucleus 17: 155, 1940.*

Effects of salts on the physical properties of sodium thymonucleate. *Proc. Am. Soc. Biol. Chemists, 1940.*

Sulfhydryl groups in the liver nucleoprotein of the calf and rabbit. *Proc. Am. Soc. Biol. Chemists, 1940.*

Chemical studies on the components of normal and neoplastic tissues. I. Viscosity and streaming birefringence of sodium thymonucleate. *J. Nat. Cancer Inst. 1: 77, 1940.* (With W. V. Jenrette.)

Chemical studies on the components of normal and neoplastic tissues. II. The nucleoprotein fraction of normal animal liver. *J. Nat. Cancer Inst. 1: 91, 1940.* (With W. V. Jenrette.)

Chemical studies on the components of normal and neoplastic tissues. III. The composition and amphoteric properties of the nucleoprotein fraction of the Jensen rat sarcoma. *J. Nat. Cancer Inst. 1: 367, 1940.* (With J. W. Thompson and W. V. Jenrette.)

Chemical studies on the components of normal and neoplastic tissues. IV. The melanin-containing pseudoglobulin of the malignant melanoma of mice. *J. Nat. Cancer Inst. 1: 377, 1940.* (With F. C. Turner and W. V. Jenrette.)

Sulfhydryl groups of serum albumin, serum, and milk. *J. Biol. Chem. 136: 795, 1940.*

Chemical studies on the components of normal and neoplastic tissues. V. The relative arginase activity of certain tumors and normal control tissues. *J. Nat. Cancer Inst. 1: 687, 1941.* (With W. V. Jenrette, G. B. Mider, and J. White.)

The relative arginase activity of certain tumors and normal control tissues. *J. Biol. Chem. 137: 795, 1941.* (With W. V. Jenrette, G. B. Mider, and J. White.)

The depolymerization of thymonucleic acid by an enzyme system in normal and cancerous hepatic and mammary tissues and in the milk and sera of several species. *J. Nat. Cancer Inst. 1: 845, 1941.* (With W. V. Jenrette.)

Physical changes in thymonucleic acid induced by proteins, salts, tissue extracts, and ultraviolet irradiation. *Cold Spring Harbor Symp. Quant. Biol. 9: 236, 1941.*

The relative activity of xanthine dehydrogenase, catalase, and amylase in normal and cancerous hepatic tissues of the rat. *J. Nat. Cancer Inst. 2: 17, 1941.* (With W. V. Jenrette and J. White.)

Effects of ultraviolet radiation on sodium thymonucleate. *J. Nat. Cancer Inst. 2: 23, 1941.* (With A. Hollaender and W. V. Jenrette.)

The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. *J. Biol. Chem. 141: 327, 1941.* (With W. V. Jenrette and J. White.)

The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. *J. Nat. Cancer Inst. 2: 283, 1941.* (With W. V. Jenrette and J. White.)

The relative enzymatic activity of certain mouse tumors and normal control tissues. *J. Nat. Cancer Inst. 2: 293, 1941.* (With W. V. Jenrette, G. B. Mider, and H. B. Andervont.)

Ribonuclease and thymonucleodepolymerase. *J. Nat. Cancer Inst. 2: 301, 1941.* (With W. V. Jenrette.)

Note on the composition of the nucleoprotein fraction of normal liver and of the transplanted hepatic tumor in the rat. *J. Nat. Cancer Inst. 2: 305, 1941.* (With W. V. Jenrette and J. White.)

The liver catalase activity of tumor-bearing mice and the effect of spontaneous regression and of removal of certain tumors. *J. Nat. Cancer Inst. 2: 345, 1942.* (With H. B. Andervont.)

A method of evaluating thymonucleodepolymerase activity in normal and tumor tissues. *J. Nat. Cancer Inst. 2: 357, 1942.*

Distribution of acid and alkaline phosphatase in tumors, normal tissues, and the tissues of tumor-bearing rats and mice. *J. Nat. Cancer Inst. 2: 511, 1942.*

Titration of the liver catalase activity of normal and of tumor-bearing rats and mice. *J. Nat. Cancer Inst. 2: 525, 1942.*

The reactivity of porphyrindin in the presence of denaturated proteins. *J. Biol. Chem. 142: 175, 1942.* (With W. V. Jenrette.)

Kidney and blood catalase activity of tumor-bearing animals. *J. Nat. Cancer Inst. 2: 589, 1942.* (With J. W. Thompson and H. B. Andervont.)

Note on the enzymatic activity of a transplanted adenocarcinoma of the glandular stomach of a mouse. *J. Nat. Cancer Inst. 2: 631, 1942.* (With H. L. Stewart.)

Comparative enzymatic activity of transplanted hepatomas and of normal, regenerating and fetal liver. *J. Nat. Cancer Inst. 3: 7, 1942.* (With J. E. Edwards, H. B. Andervont, and J. White.)

Sulfhydryl groups in normal and tumorous hepatic tissue extracts before and after addition of salts. *J. Nat. Cancer Inst. 3: 61, 1942.*

The creatine and creatinine content of transplanted hepatomas and of normal and regenerating liver. *J. Nat. Cancer Inst. 3: 287, 1942.*

The incubation of citrulline and ammonia with normal and neoplastic hepatic tissues. *J. Nat. Cancer Inst. 3: 293, 1942.*

Electrophoretic patterns, colloid osmotic pressure and viscosity of serum denatured by ultraviolet radiation. *J. Biol. Chem. 146: 663, 1942.* (With B. D. Davis and A. Hollaender.)

Further studies of the liver catalase activity of tumor-bearing animals. *J. Nat. Cancer Inst. 3: 397, 1943.*

Note on the copper content of the tissues of tumor-bearing animals. *J. Nat. Cancer Inst. 3: 405, 1943.* (With J. W. Thompson.)

Tumor enzymology. *J. Nat. Cancer Inst. 3: 419, 1943.*

Recent progress in tumor enzymology. In *Advances Enzymology, Vol. 3, 1943, p. 315.*

Degradation of cystine by normal liver but not by transplanted hepatomas. *J. Nat. Nat. Cancer Inst. 3: 491, 1943.*

Peptides, peptidases and diketopiperazines. Addendum to the Chemistry of the Amino Acids and Proteins (Schmidt, C. L. A., ed.). *Springfield, Ill., Charles C Thomas, Chapt. 6, 1943.*

Deploymerases for yeast and for thymus nucleic acids in normal and neoplastic tissues. *J. Nat. Cancer Inst. 4: 55, 1943.*

Note on the colloid osmotic pressure of the serums of rats bearing the transplanted Jensen sarcoma. *J. Nat. Cancer Inst. 4: 63, 1943.* (With J. W. Thompson.)

The colloid osmotic pressure of mixtures of protein and thymus nucleate. *J. Biol. Chem. 150: 107, 1943.*

Friedrich Miescher, 1844–1895, founder of nuclear chemistry. *Scientific Monthly 57: 523, 1943.*

Enzymatic activity of normal adult, regenerating, fetal and neoplastic tissues of the rat. *J. Nat. Cancer Inst. 4: 271, 1943.* (With J. W. Thompson.)

Range in activity of several enzymes in normal and neoplastic tissues of mice. *J. Nat. Cancer Inst. 4: 275, 1943.* (With J. W. Thompson.)

Note on the liver catalase activity of pregnant mice and of mice bearing growing embryonic implants. *J. Nat. Cancer Inst. 4: 283, 1943.* (With H. B. Andervont.)

A transplantable osteogenic sarcoma originating in a C3H mouse. *J. Nat. Cancer Inst. 4: 389, 1944.* (With M. K. Barrett, A. J. Dalton, and J. E. Edwards.)

Nucleoproteins. In *Advances Protein Chem. 3: 209, 1944.*

The chemistry of protein denaturation. *Chem. Revs. 34: 157, 1944.* (With H. Neurath, F. W. Putnam, and J. C. Erickson.)

Book review: Biochemie der Tumoren by V. Euler and B. Skarzynski. Stuttgart, Germany, Ferdinand Enke, 1942. In *Cancer Res. 4: 400, 1944.*

The chemistry of the proteins and amino acids. *Ann. Rev. Biochem. 13: 117, 1944.* (With H. Neurath.)

Degradation of cystine peptides by tissues—a new aspect of protein catabolism. *Science 101: 19, 1944.* (With F. M. Leuthardt.)

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Cystine and cysteine in the water-extractable proteins of rat and rabbit tissues. *J. Biol. Chem. 156: 349, 1944.* (With F. M. Leuthardt.)

Chemistry of mammary tumors in the mouse. *In* Am. Assoc. Adv. Sc. Monograph (Staff of the National Cancer Institute). *Washington, D.C., A.A.A.S., 1944.*

Esterase activity of blood serum of four strains of mice. *J. Nat. Cancer Inst. 5: 29, 1944.* (With M. B. Shimkin and H. B. Andervont.)

Esterase (butyric esterase) activity of normal and neoplastic tissues of the mouse. *J. Nat. Cancer Inst. 5: 31, 1944.*

Comparative oxidase activity of melanotic and amelanotic melanomas. *J. Nat. Cancer Inst. 5: 35, 1944.* (With G. H. Algire.)

Note on the cystine oxidase activity in normal and neoplastic tissues of the mouse. *J. Nat. Cancer Inst. 5: 39, 1944.* (With F. M. Leuthardt.)

Chemical studies on human cancer. I. Cytochrome oxidase, cytochrome *C*, and copper in normal and neoplastic tissues. *J. Nat. Cancer Inst. 5: 55, 1944.* (With J. Werne, A. B. Eschenbrenner, and F. M. Leuthardt.)

Sulfur distribution in extracts of normal and neoplastic animal tissues. *J. Nat. Cancer Inst. 5: 111, 1944.* (With F. M. Leuthardt.)

The degradation of cystine peptides by tissues. I. Exocystine desulfurase and dehydropeptidase in rat liver extracts. *J. Nat. Cancer Inst. 5: 209, 1944.* (With F. M. Leuthardt.)

The degradation of cystine peptides by tissues. II. Distribution of exocystine desulfurase and dehydropeptidase in tissue extracts of various species. *J. Nat. Cancer Inst. 5: 223, 1944.*

Enzymes in normal and neoplastic animal tissues. *In* Research Conf. on Cancer (1944). *Washington, D.C., Am. Assoc. Adv. Sc., occasional publ., 1945, pp. 192–222.*

Biochemistry of malignant tissues. *Ann. Rev. Biochem. 14: 643, 1945.*

Book Review: Advances in Enzymology and Related Subjects of Biochemistry (Nord, F. F., and Werkman, C. H., eds.). New York, Interscience Publishers, Inc., Vol. 4, 1944. In *Cancer Res. 5: 62, 1945.*

Degradation of cystine peptides by tissues. III. Absence of exocystine desulfurase and dehydropeptidase in tumors. *J. Nat. Cancer Inst. 5: 249, 1945.* (With F. M. Leuthardt.)

Effect of nucleates on the rate of methylene blue decolorization in tissue extracts. *J. Biol. Chem. 157: 753, 1945.* (With H. W. Chalkley.)

The influence of nucleic acid on dehydrogenase systems—a contribution to the problem of gene mechanism. *Ann. Missouri Bot. Gar. 32: 179, 1945.* (With H. W. Chalkley.)

Book Review: Outline of the Amino Acids and Proteins (Sahyun, M., ed.). New York, Rheinhold Pub. Corp., 1944. In *J. Am. Chem. Soc. 67: 885, 1945.*

Desaminases for ribosenucleic and desoxyribosenucleic acids. *J. Nat. Cancer Inst. 6: 61, 1945.* (With H. W. Chalkley.)

Desaminases for ribosenucleic and desoxyribosenucleic acids. *Arch. Biochem. 7: 451, 1945.* (With H. W. Chalkley.)

Effect of nucleates on dehydrogenase systems. *J. Nat. Cancer Inst. 6: 119, 1945.* (With H. W. Chalkley.)

Effect of sodium bicarbonate, pyruvate, and diphosphopyridine nucleotide (DPN) on dehydrogenase activity. *J. Nat. Cancer Inst. 6: 143, 1945.* (With H. W. Chalkley.)

Effect of bicarbonate and diphosphopyridine nucleotide (DPN), on dehydrogenase activity in liver extracts. *J. Biol. Chem. 160: 371, 1945.* (With H. W. Chalkley.)

Book Review: Advances in Enzymology and Related Subjects of Biochemistry (Nord, F. F., and Werkman, C. H., eds.). New York, Interscience Publishers Inc., 1945 Vol. 5. In *Cancer Res. 6: 46, 1946.*

Tumor enzymology. *Actualite Rev. Med. Brussels, 1946.*

Degradation of cystine peptides by tissues. IV. Dehydropeptidase activity in normal and neoplastic tissues. *J. Nat. Cancer Inst. 6: 197, 1946.* (With F. M. Leuthardt.)

Enzymatic hydrolysis of benzoylarginineamide in normal and neoplastic tissues. *J. Nat. Cancer Inst. 6: 203, 1946.* (With F. M. Leuthardt.)

Note on some aspects of the effect of nucleates in primary and transplanted rat hepatomas. *J. Nat. Cancer Inst. 6: 207, 1946.* (With H. W. Chalkley.)

Protective effect of thymus nucleate on the heat coagulation of proteins. *J. Nat. Cancer Inst. 6: 219, 1946.* (With C. E. Carter.)

Note on the enzymatic activity of normal and neoplastic tissues of the rat. *J. Nat. Cancer Inst. 6: 317, 1946.* (With F. M. Leuthardt.)

Dehydropeptidase activity in tissues. *J. Biol. Chem. 162: 175, 1946.* (With F. M. Leuthardt.)

Thymus nucleate and the heat coagulation of egg albumin. *Proc. Am. Soc. Biol. Chemists, 1946.* (With C. E. Carter.)

Thymus nucleate and the heat coagulation of tissue extracts. *Proc. Am. Soc. Biol. Chemists, 1946.* (With C. E. Carter.)

Neutral β-glycerophosphatase activity in normal and neoplastic tissues. *J. Nat. Cancer Inst. 7: 1, 1946.* (With F. M. Leuthardt.)

Enzymatic desamination and dephosphorylation of ribosenucleic and desoxyribosenucleic acids. *J. Nat. Cancer Inst. 7: 9, 1946.* (With C. E. Carter, H. W. Chalkley, and F. M. Leuthardt.)

Studies on the enzymatic degradation of nucleic acids. *J. Nat. Cancer Inst. 7: 29, 1946.* (With C. E. Carter.)

Book Review: Advances in Protein Chemistry (Edsall, J. T., and Anson, M. L., eds.). New York, Academic Press, Inc., Vol. 2, 1945. In *Science 103: 210, 1946.*

The enzymatic desamination, dephosphorylation and degradation of nucleic acids to dialyzable substances. *Arch. Biochem. 11: 307, 1946.*

Activity of phosphatases in fresh and dialyzed extracts of normal mouse liver and of mouse hepatoma. *J. Nat. Cancer Inst. 7: 47, 1946.* (With C. E. Carter and F. M. Leuthardt.)

Spectrophotometric determination of dehydropeptidase activity in normal and neoplastic tissues. *J. Nat. Cancer Inst. 7: 51, 1946.* (With C. E. Carter.)

Influence of α-keto acids on the desamidation of amino acid amines. *J. Nat. Cancer Inst. 7: 57, 1946.* (With C. E. Carter.)

A spectrophotometric method for the determination of dehydropeptidase activity. *J. Biol. Chem. 165: 725, 1946.* (With C. E. Carter.)

The influence of α-keto acids on the desamidation of amino acid amines. *J. Biol. Chem. 165: 741, 1946.* (With C. E. Carter.)

Enzymatic desamidation of glutamine in the presence of pyruvate furnished by concomitant reactions. *J. Nat. Cancer Inst. 7: 269, 1947.* (With J. M. Gonçalves.)

Studies on the effect of pyruvate on the desamidation of glutamine, asparagine and related compounds. *J. Nat. Cancer Inst. 7: 275, 1947.* (With V. E. Price.)

Desamidation of amino acid amides in rat-liver extracts of varying concentrations. *J. Nat. Cancer Inst. 7: 281, 1947.* (With J. M. Gonçlaves and V. E. Price.)

Desamidation of glutamine and asparagine in normal and neoplastic hepatic tissues. *J. Nat. Cancer Inst. 7: 285, 1947.* (With M. Errera.)

Acceleration of enzymatic desamidation of glutamine by several inorganic anions. *J. Nat. Cancer Inst. 7: 433, 1947.* (With C. E. Carter.)

Effects of acidity and of heating on the capacity of rat liver extracts to desamidate glutamine and asparagine. *J. Nat. Cancer Inst. 7: 437, 1947.* (With M. Errera.)

Enzymatic hydrolysis of acetyldehydroalanine. *J. Nat. Cancer Inst. 7: 443, 1947.* (With J. M. Gonçalves and V. E. Price.)

Enzymatic hydrolysis of α,α-diglycylaminopropionic acid hydrochloride. *J. Nat. Cancer Inst. 8: 25, 1947.* (With J. M. Gonçalves.)

Note on the splitting off of α,α-diglycylaminopropionic acid in extracts of rat hepatic tissues. *J. Nat. Cancer Inst. 8: 29, 1947.* (With M. de Mingo.)

Relative amount of ammonia evolved from digests of isomeric peptides with aqueous tissue extracts. *J. Nat. Cancer Inst. 8: 31, 1947.* (With J. M. Gonçalves and V. E. Price.)

Presence of dehydropeptidase I and II activity in plant material. *J. Nat. Cancer Inst. 8: 35, 1947.* (With F. M. Leuthardt.)

Spectral and chemical properties of pyruvoylglycine. *J. Nat. Cancer Inst. 8: 39, 1947.* (With M. Errera.)

Enzymatic degradation of ribosenucleic and desoxyribosenucleic acids with an addendum on the effect of nucleates on the heat stability of proteins. *Cold Spring Harbor Symp. Quant. Biol. 12: 64–94, 1947.* (With C. E. Carter and H. W. Chalkley.)

Effects of x radiation on thymus nucleic acid. *Science 105: 263–264, 1947.* (With B. Taylor and A. Hollaender.)

A new synthesis of chloroacetyldehydroalanine. *Arch. Biochem. 14: 249, 1947.* (With V. E. Price.)

Dehydropeptidase activity in certain animal and plant tissues. *J. Biol. Chem. 171: 477, 1947.* (With V. E. Price.)

Enzymatic degradation of ribosenucleic and desoxyribosenucleic acids with addendum on effect of nucleates on heat stability proteins. *Fed. Proc. 6: 488–499, 1947.*

Ammonia nitrogen from digests of isomeric peptides in kidney homogenate digests. *Science 106: 369, 1947.* (With J. M. Gonçalves and V. E. Price.)

Note on the ultraviolet absorption spectra of keto acids and of keto acid peptides. *Arch. Biochem. 15: 445, 1947.* (With M. Errera.)

Effect of various keto acids on desamidation of glutamine. *Arch. Biochem. 15: 449, 1947.* (With M. Errera.)

Enzymatic hydrolysis of homologous amino acid amides in normal and neoplastic tissues. *J. Nat. Cancer Inst. 8: 71, 1947.* (With M. Errera.)

Note on the benzoylarginineamidase activity in extracts of rat liver and hepatoma. *J. Nat. Cancer Inst. 8: 77, 1947.* (With F. M. Leuthardt.)

Biochemistry of Cancer. *New York, Academic Press, Inc., 1947, 400 pp.*

Enzymatic hydrolysis of α,α-di(glycylamino)propionic acid. *Arch. Biochem. 16: 1, 1948.* (With J. M. Gonçalves.)

Effects of X-radiation on sodium thymus nucleate. *Arch Biochem. 16: 19, 1948.* (With B. Taylor and A. Hollaender.)

Chemistry of Melanomas. *Special Publ. New York Acad. Sc. 4: 433, 1948.*

N-acylated and N-methylated glycyldehydroalanine. *J. Biol. Chem. 173: 337, 1948.* (With V. E. Price.)

Further studies on α,α-di(acylamino)propionic acids. *Arch. Biochem. 17: 51, 1948.* (With V. E. Price and M. Errera.)

Effect of phosphate and other anions on the enzymatic desamidation of various amides. *Arch. Biochem. 17: 105, 1948.* (With F. M. Leuthardt.)

Effect of added phosphate on glutamine desamidation in tumors. *J. Nat. Cancer Inst. 8: 161, 1948.* (With F. M. Leuthardt.)

Dehydropeptidase activity of normal and pathological human sera. *J. Nat. Cancer Inst. 8: 169, 1948.* (With A. Meister.)

Dehydropeptidases. *Adv. Enzymol. 8: 117, 1948.*

Ammonia formation from cystine peptides and dehydropeptides. *Arch. Biochem. 18: 377, 1948.* (With F. M. Leuthardt.)

Acetylated dehydroamino acids. *Arch. Biochem. 18: 383, 1948.* (With V. E. Price.)

Addition products of dehydropeptides. *Arch. Biochem. 19: 467, 1948.* (With I. Z. Eiger.)

Enzymatic hydrolysis of 2,4-diketo acids. *J. Biol. Chem. 175: 573, 1948.* (With A. Meister.)

Studies on the possible multiple nature of dehydropeptidase I. *J. Biol. Chem. 175: 953, 1948.* (With V. E. Price and F. M. Leuthardt.)

Derivatives of α,α-di(glycylamino)propionic acid. *J. Biol. Chem. 175: 963, 1948.* (With V. E. Price.)

Enzymatic hydrolysis of analogous saturated and unsaturated peptides. *J. Biol. Chem. 175: 969, 1948.* (With V. E. Price.)

Carcinogens. *Encyclopedia Chem. Technol., Vol. 3, 1949.*

Phosphate-activated glutaminase in kidney and other tissues. *J. Biol. Chem. 178: 495, 1949.* (With M. Errera.)

Preparation of L- and D-alanine by enzymatic resolution of acetyl-DL-alanine. *J. Biol. Chem. 178: 503, 1949.* (With P. J. Fodor and V. E. Price.)

Keto acid-activated glutaminase and asparaginase. *J. Biol. Chem. 178: 695, 1949.* (With V. E. Price.)

The resolution of several racemic amino acids. *J. Biol. Chem. 179: 1169, 1949.* (With V. E. Price and J. B. Gilbert.)

Enzymatic hydrolysis of saturated and unsaturated tripeptides. *J. Biol. Chem. 180: 193, 1949.* (With P. J. Fodor and V. E. Price.)

Effect of anions on the non-enzymatic desamidation of glutamine. *J. Biol. Chem. 180: 209, 1949.*

Resolution of racemic phenylalanine, tyrosine, and tryptophan. *J. Biol. Chem. 180: 473, 1949.* (With J. B. Gilbert and V. E. Price.)

The separation of dehydropeptidase and analogous L- and D-peptidases. *J. Biol. Chem. 181: 535, 1949.* (With V. E. Price, A. Meister, and J. B. Gilbert.)

Separation of enzymatic activities toward chloroacetylalanine, chloroacetylalanylglycine and glycylalanine. *J. Biol. Chem. 181: 549, 1949.* (With P. J. Fodor.)

Some biochemical aspects of tumors. In *Proc. Papal Acad. Sc., Rome, 1949.*

Dehydropeptidase activity in tumors. *J. Nat. Cancer Inst. 9: 389, 1949.* (With F. M. Leuthardt.)

The neoplastic transformation as a biological fractionation of related enzyme systems. *J. Nat. Cancer Inst. 10: 271, 1949.* (With P. J. Fodor and F. M. Leuthardt.)

1950–1954

Enzymatic resolution of racemic lysine, norleucine, norvaline, and α-aminobutric acid. *J. Biol. Chem. 182: 451, 1950.* (With J. B. Gilbert and P. J. Fodor.)

Effect of thymus nucleate on the thermal coagulation of albumin solutions. *J. Biol. Chem. 182: 457, 1950.* (With M. L. Hoyer.)

A resolution of histidine, cystine and alanine by asymmetric enzymatic hydrolysis of the racemic amides. *J. Biol. Chem. 184: 55, 1950.* (With L. Levintow and V. E. Price.)

The enzymatic hydrolysis of L- and D-amino acid amides by mushroom preparations. *Arch. Biochem. 26: 92, 1950.* (With V. E. Price, L. Levintow, and R. B. Kingsley.)

Preparation and enzymatic hydrolysis of three new homologous dehydropeptides. *J. Biol. Chem. 184: 633, 1950.* (With L. Levintow, S-C. J. Fu, and V. E. Price.)

Configuration of isomeric allothreonines by enzymatic resolution. *J. Am. Chem. Soc. 72: 2812, 1950.* (With L. Levintow.)

The present status of cancer research. *Sc. Counselor 13: 135, 1950.*

Preparation and properties of α,α-di(acylamino)aliphatic acids. *Arch. Biochem. 28: 440, 1950.* (With S-C. J. Fu, L. Levintow, and V. E. Price.)

Preparation of D-ornithine, D-citrulline and D-arginine. *J. Biol. Chem. 188: 643, 1951.* (With L. Levintow.)

Preparation of the four stereoisomers of isoleucine. *J. Biol. Chem. 188: 647, 1951.* (With L. Levintow, C. G. Baker, and J. White.)

A resolution of glutamic acid. *Arch. Biochem. 31: 77, 1951.* (With L. Levintow and R. B. Kingsley.)

Alkali-catalyzed ring closure of pyruvoylglycine. *Arch. Biochem. 31: 83, 1951.* (With S-C. J. Fu and V. E. Price.)

Further studies on pyruvoyl amino acids. *Arch. Biochem. 32: 365, 1951.* (With S-C. J. Fu.)

Cation activation of desoxyribonuclease. *Arch. Biochem. 32: 414, 1951.* (With T. Miyaji.)

Optical purity of amino acid enantiomorphs. *J. Biol. Chem. 192: 535, 1951.* (With A. Meister, L. Levintow, and R. B. Kingsley.)

An enzymatic resolution of proline. *J. Biol. Chem. 193: 81, 1951.* (With D. Hamer.)

Tumor enzymology. *In* The Enzymes (Sumner, J. B., and Myrbäck, K., eds.). *New York, Academic Press, Inc., Vol. 2, Chapt. 2, 1952.* (With A. Meister.)

Preparation of the optical isomers of analogous cyclohexyl- and phenyl-substituted amino acids. *J. Am. Chem. Soc. 74: 551, 1952.* (With D. Rudman and A. Meister.)

Specificity of amino acid acylases. *J. Biol. Chem. 194: 455, 1952.* (With S. M. Birnbaum, L. Levintow, and R. B. Kingsley.)

Effect of cancer on liver enzymes. *J.A.M.A. 148: 697, 1952.*

Stereoisomers of hydroxyproline. *J. Biol. Chem. 195: 383, 1952.* (With D. S. Robinson.)

Glycyl dehydropeptides of leucine, valine, and isoleucine. *J. Biol. Chem. 195: 849, 1952.* (With A. Meister.)

Preparation of the optical isomers of arginine, histidine and S-benzylcysteine by asymmetric enzymatic hydrolysis of their acetyl derivatives. *Arch. Biochem. 39: 108, 1952.* (With S. M. Birnbaum.)

Optical enantiomorphs of isovaline. *J. Am. Chem. Soc. 74: 4701, 1952.* (With C. G. Baker, S-C. J. Fu, S. M. Birnbaum, and H. A. Sober.)

Optical enantiomorphs of α,β-diaminopropionic acid. *J. Biol. Chem. 198: 335, 1952.* (With S. M. Birnbaum, R. J. Koegel, and S-C J. Fu.)

Enzymatic susceptibility of corresponding chloroacetyl- and glycyl-L-amino acids. *J. Biol. Chem. 198: 507, 1952.* (With K. R. Rao, S. M. Birnbaum, and R. B. Kingsley.)

Method of enzymatic resolution of amino acids. *U.S. Patent 2,616,828, 1952.* (With L. Levintow.)

Enzymatic resolution of α,γ-diaminobutyric acid. *J. Biol. Chem. 199: 207, 1952.* (With S-C. J. Fu, K. R. Rao, and S. M. Birnbaum.)

L-Isoleucine, D-isoleucine, L-alloisoleucine and D-alloisoleucine. *Biochem. Prep. 3: 84, 1953.* (With S. M. Birnbaum and L. Levintow.)

Optical enantimorphs of homoserine and homocystine. *Arch. Biochem. 42: 212, 1953.* (With S. M. Birnbaum.)

Conversion of β-chloroacetyl-L-diaminopropionic acid to L-2-ketopiperazine-5-carboxylic acid. *J. Biol. Chem. 201: 547, 1953.* (With R. J. Koegel, S. M. Birnbaum, C. G. Baker, and H. A. Sober.)

Purification and properties of an aminopeptidase from kidney cellular particulates. *J. Biol. Chem. 202: 1, 1953.* (With D. S. Robinson and S. M. Birnbaum.)

Optical enantiomorphs of α-aminoadipic acid. *J. Am. Chem. Soc. 75: 1994, 1953.* (With S. M. Birnbaum and M. C. Otey.)

Enzymatic susceptibility of comparable N-acylated L-, D- and dehydroamino acids. *J. Biol. Chem. 203: 1, 1953.* (With K. R. Rao and S. M. Birnbaum.)

Resolution of the racemic α-amino derivatives of heptylic, caprylic, nonylic, decylic, and undecylic acids. *J. Biol. Chem. 203: 333, 1953.* (With S. M. Birnbaum and S-C. J. Fu.)

Optical and enzymatic characterization of amino acids. *J. Biol. Chem. 204: 307, 1953.* (With S. M. Birnbaum and M. C. Otey.)

Optical enantiomorphs of tertiary leucine. *J. Biol. Chem. 205: 221, 1953.* (With N. Izumiya, S-C. J. Fu, and S. M. Birnbaum.)

Erwin Brand: 1891–1953. *Science 119: 144, 1954.* (With O. E. Reynolds.)

Solubilized kidney glutaminase I. *Arch. Biochem. 49: 245, 1954.* (With M. C. Otey and S. M. Birnbaum.)

Biochemistry of Cancer, 2d ed. *New York, Academic Press, Inc., 1954.*

Peptidases in renal and hepatic tissues. *Bull. Israeli Acad. Sc. 4: 6, 1954.* (With S. M. Birnbaum.)

The resolution of racemic α-amino acids. *Advances Protein Chem. 9: 121, 1954.*

Studies on polycysteine peptides and proteins. I. Isomeric cystinylcystine peptides. *Arch. Biochem. 52: 203, 1954.* (With N. Izumiya.)

Studies on polycysteine peptides and proteins. II. Apparent dissociation constants, and ultraviolet and infrared absorption spectra of isomeric cystinylcystine peptides. *Arch. Biochem. 53: 501, 1954.* (With M. C. Otey.)

Influence of optically active acyl groups on the enzymatic hydrolysis of N-acylated-L-amino acids. *J. Am. Chem. Soc. 76: 6054, 1954.* (With S-C. J. Fu and S. M. Birnbaum.)

1955–1959

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. I. Preparation of the four optical isomers of α-aminotricarballylic acid and their conversion to the corresponding isocitric acid lactones. *J. Am. Chem. Soc. 77: 707, 1955.* (With N. Izumiya, M. Winitz, and S. M. Birnbaum.)

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. II. Contribution of the asymmetric α and β carbon atoms to the molar rotations of the diastereomeric α-aminotricarballylic acids and corresponding isocitric acids. *J. Am. Chem. Soc. 77: 716, 1955.* (With M. Winitz and S. M. Birnbaum.)

Separation of the three isomeric components of synthetic α,ϵ-diaminopimelic acid. *J. Am. Chem. Soc. 77: 1916, 1955.* (With E. Work, S. M. Birnbaum, and M. Winitz.)

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. III. Configuration of the β-asymmetric center of isoleucine. *J. Am. Chem. Soc. 77: 3106, 1955.* (With M. Winitz and S. M. Birnbaum.)

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. IV. Rotary dispersion of the asymmeteric α- and ω-carbon atoms of several diastereomeric amino acids. *J. Am. Chem. Soc. 77: 3112, 1955.* (With M. C. Otey, M. Winitz, and S. M. Birnbaum.)

Saturation of acetyldehydroalanine with benzylamine. *J. Am. Chem. Soc. 77: 4412, 1955.* (With S-C. J. Fu.)

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. V. Infrared spectra. *J. Am. Chem. Soc. 77: 5708, 1955.* (With R. J. Koegel, M. Winitz, S. M. Birnbaum, and R. A. McCallum.)

Studies on carcinogenic amines. I. L- and D-Amino acid derivatives of 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl, and their susceptibility to hydrolysis by tissue homogenates. *J. Am. Chem. Soc. 77: 5721, 1955.* (With M. Winitz and S. M Birnbaum.)

The *in vivo* effect on liver catalase by a tumor. *J. Nat. Cancer Inst. 15: 1603, 1955.*

Studies on polycysteine peptides and proteins. III. Configurations of the peptides of L-cystine obtained by oxidation of L-cysteinyl-L-cysteine. *J. Am. Chem. Soc. 78: 373, 1956.* (With R. Wade and M. Winitz.)

A simplified preparation of D-arginine. *Arch. Biochem. 60: 496, 1956.* (With S. M. Birnbaum and M. Winitz.)

Book Review: Annual Review of Biochemistry (Luck, J. M., Loring, H. S., and Mackinney, G., eds.). Stanford, Calif. Annual Reviews, Inc., 1955. In *J. Am. Chem. Soc. 78: 1075, 1956.*

Studies on disastereoisomeric α-amino acids and corresponding α-hydroxy acids. VI. Rotatory dispersion of copper complexes. *J. Am. Chem. Soc. 78: 1602, 1956.* (With N. Izumiya, M. Winitz, and S. M. Birnbaum.)

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. VII. Influence of β-configuration on enzymic susceptibility. *J. Am. Chem. Soc. 78: 2423, 1956.* (With M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, and C. G. Baker.)

Some biochemical characteristics of morphologically separable cancers. *Cancer Res. 16: 641, 1956.*

Cobalt ion activation of renal acylase I. *J. Am. Chem. Soc. 78: 4636, 1956.* (With R. Marshall and S. M. Birnbaum.)

Book Review: Currents in Biochemical Research 1956 (Green, D. E., ed.). New York Interscience Publishers Inc., 1956. In *J. Am. Chem. Soc. 78: 5455, 1956.*

Collected Lectures in Japan (I–IV, incl.) 1956. Auspices of Japanese Biochemical Society.

Studies on the metabolism of amino acids and related compounds *in vivo*. I. Toxicity of essential amino acids, individually and in mixtures, and the protective effect of L-arginine. *Arch. Biochem. 58: 253, 1955; 64: 319, 1956.* (With P. Gullino, M. Winitz, S. M. Birnbaum, J. Cornfield, and M. C. Otey.)

Studies on the metabolism of amino acids and related compounds *in vivo*. II. Effect of toxic doses of essential amino acids on blood sugar, liver glycogen, and muscle glycogen levels. *Arch. Biochem. 64: 333, 1956.* (With M. Winitz, P. Gullino, and S. M. Birnbaum.)

Studies on the metabolism of amino acids and related compounds *in vivo*. III. Prevention of ammonia toxicity by arginine and related compounds. *Arch. Biochem. 59: 302, 1955; 64: 342, 1956.* (With M. Winitz, P. Gullino, S. M. Birnbaum, and M. C. Otey.)

Studies on the metabolism of amino acids and related compounds *in vivo*. IV. Blood ammonia and urea levels following intraperitoneal administration of amino acids and ammonium acetate, and the effect of arginine thereon. *Arch. Biochem. 62: 236, 1956; 64: 355, 1956.* (With J. P. du Ruisseau, M. Winitz, and S. M. Birnbaum.)

Studies on the metabolism of amino acids and related compounds *in vivo*. V. Effects of combined administration of nonprotective compounds and subprotective levels of L-arginine·HCl on ammonia toxicity in rats. *Arch. Biochem. 64: 368, 1956.* (With M. Winitz, J. P. du Ruisseau, M. C. Otey, and S. M. Birnbaum.)

A water-soluble synthetic diet. *Arch. Biochem. 63: 266, 1956.* (With S. M. Birnbaum and M. Winitz.)

The percarbobenzoxylation of L-arginine. *Arch. Biochem. 65: 573, 1956.* (With L. Zervas and M. Winitz.)

Studies on arginine peptides. I. Intermediates in the synthesis of N-terminal and C-terminal arginine peptides. *J. Org. Chem. 22: 1515, 1957.* (With L. Zervas and M. Winitz.)

Preparation and properties of the isomeric forms of α-amino- and α,ε-diaminopimelic acid. *J. Am. Chem. Soc. 79: 648, 1957.* (With R. Wade, S. M. Birnbaum, M. Winitz, and R. J. Koegel.)

Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. VIII. Configuration of the isomeric octopines. *J. Am. Chem. Soc. 79: 652, 1957.* (With N. Izumiya, R. Wade, M. Winitz, M. C. Otey, S. M. Birnbaum, R. J. Koegel.)

Studies on the metabolism of amino acids and related compounds *in vivo*. VI. Free amino acid levels in the tissues of rats protected against ammonia toxicity. *Arch. Biochem. 68: 161, 1957.* (With J. P. du Ruisseau, M. Winitz, and S. M. Birnbaum.)

Studies on the metabolism of amino acids and related compounds *in vivo*. VII. Ammonia toxicity in partially hepatectomized rats and the effect of L-arginine·HCl thereon. *Arch. Biochem. 71: 458, 1957.* (With J. P. du Ruisseau, M. Winitz, and S. M. Birnbaum.)

ε-Lysine acylase. *Arch. Biochem. 69: 56, 1957.* (With W. K. Paik, L. Bloch-Frankenthal, S. M. Birnbaum, and M. Winitz.)

Preparation and properties of the isomeric forms of cystine and S-benzylpenicillamine. *J. Am. Chem. Soc. 79: 4538, 1957.* (With R. Marshall, M. Winitz, and S. M. Birnbaum.)

Studies on diastereomeric α-amino acids and corresponding α-hydroxy acids. IX. Configuration of the isomeric γ-hydroxy-glutamic acids. *J. Am. Chem. Soc. 79: 6192, 1957.* (With L. Benoiton, M. Winitz, and S. M. Birnbaum.)

Quantitative nutritional studies with water-soluble, chemically defined diets. I. Growth, reproduction and lactation in rats. *Arch. Biochem. 72: 396, 1957.* (With S. M. Birnbaum, M. Winitz, and M. C. Otey.)

Quantitative nutritional studies with water-soluble, chemically defined diets. II. Nitrogen balance and metabolism. *Arch. Biochem. 72: 417, 1957.* (With S. M. Birnbaum and M. Winitz.)

Quantitative nutritional studies with water-soluble, chemically defined diets. III. Individual amino acids as sources of "nonessential" nitrogen. *Arch. Biochem. 72: 428, 1957.* (With S. M. Birnbaum and M. Winitz.)

Quantitative nutritional studies with water-soluble, chemically defined diets. IV. Influence of various carbohydrates on growth, with special reference to D-glucosamine. *Arch. Biochem. 72: 437, 1957.* (With M. Winitz and S. M. Birnbaum.)

Quantitative nutritional studies with water-soluble, chemically defined diets. V. Role of the isomeric arginines in growth. *Arch. Biochem. 72: 448, 1957.* (With M. Winitz and S. M. Birnbaum.)

A synthesis of L-arginyl-L-arginine. *Arch. Biochem. 75: 290, 1958.* (With L. Zervas, T. Otani, and M. Winitz.)

Studies on the metabolism of amino acids and related compounds *in vivo*. VIII. Influence of the route of administration of L-arginine·HCl on protecting rats against ammonia toxicity. *Arch. Biochem. 76: 430, 1958.* (With P. Gullino, S. M. Birnbaum, and M. Winitz.)

The use of amino acid oxidases for the small-scale preparation of the optical isomers of amino acids. *J. Am. Chem. Soc. 80: 953, 1958.* (With J. R. Parikh, M. Winitz, and S. M. Birnbaum.)

Quantitative nutritional studies with water-soluble, chemically defined diets. VI. Growth studies on mice. *Arch. Biochem. 78: 245, 1958.* (With S. M. Birnbaum, M. E. Greenstein, and M. Winitz.)

The enzymic resolution of β-methylaspartic acid with acylase II. *Arch. Biochem. 81: 434, 1959.* (With L. Benoiton, S. M. Birnbaum, and M. Winitz.)

Studies on diasteremeric α-amino acids and corresponding α-hydroxy acids. X. The preparation of β-hydroxy-β-methylaspartic acid. *J. Am. Chem. Soc. 81: 1726, 1959.* (With L. Benoiton, M. Winitz, R. F. Colman, and S. M. Birnbaum.)

Preparation and disulfide interchange reactions of unsymmetrical open-chain derivatives of cystine. *J. Am. Chem. Soc. 81: 1729, 1959.* (With L. Zervas, L. Benoiton, E. Weiss, and M. Winitz.)

Quantitative nutritional studies with water-soluble, chemically defined diets. VII. Nitrogen balance in normal and tumor-bearing rats following forced-feeding. *Arch. Biochem. 81: 439, 1959.* (With T. Sugimura, S. M. Birnbaum, and M. Winitz.)

Quantitative nutritional studies with water-soluble, chemically defined diets. VIII. The forced-feeding of diets each lacking in one essential amino acid. *Arch. Biochem. 81: 448, 1959.* (With T. Sugimura, S. M. Birnbaum, and M. Winitz.)

Studies on arginine peptides. II. Synthesis of L-arginyl-L-arginine and other N-terminal arginine dipeptides. *J. Am. Chem. Soc. 81: 2878, 1959.* (With L. Zervas, T. Otani, and M. Winitz.)

Book Review: Causation of Cancer (Boyland, E., ed.). England, Med. Dept., The British Council, 1958. (In *Brit. M. Bull. 14: 73, No. 2*) In *J. Am. Chem. Soc. 80: 6696, 1958.*

A synthesis and resolution of DL-serine. *Arch. Biochem. 83: 1, 1959.* (With S. Akabori, T. T. Otani, R. Marshall, and M. Winitz.)

Quantitative nutritional studies with water-soluble, chemically defined diets. IX. Further studies on D-glucosamine containing diets. *Arch. Biochem. 83: 521, 1959.* (With T. Sugimura, S. M. Birnbaum, and M. Winitz.)

Quantitative nutritional studies with water-soluble, chemically defined diets. X. Formulation of a nutritionally complete liquid diet. *J. Nat. Cancer Inst. 24: 211–219, 1960.* (With M. C. Otey, S. M. Birnbaum, and M. Winitz.)

Chemistry of the Amino Acids. 3 Vols. *New York, John Wiley & Sons.* To be published, 1960. (With M. Winitz.)

Metabolism of Neoplastic Tissue. XV. Oxidation of Exogenous Fatty Acids in Lettré-Ehrlich Ascites Tumor Cells [1, 2]

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SUMMARY

Lettré-Ehrlich ascites tumor cells were incubated *in vitro* in a phosphate saline medium with palmitate-1-C^{14}, and its oxidation was measured by following the incorporation of radioactivity in respiratory CO_2. Regardless of whether the labeled fatty acid was present as plasma lipide, free palmitate, or as the serum-protein complex, it was oxidized readily at slightly less than linear rates over periods up to 4 hours. Oxidation of the 3 forms was greatly depressed by glucose, to a lesser extent by galactose, and only slightly by lactate and acetate. The data indicate that fatty acids are major substrates for tumor respiration, but their oxidation is partially spared when glucose is present in adequate concentration. These findings amplify and confirm previous conclusions based on studies of endogenous fatty-acid oxidation.—J. Nat. Cancer Inst. 24: 1–12, 1960.

THE PRESENT report describes further progress in a continuing study of the nature of respiratory substrates of tumor cells and of factors which influence their participation in the respiratory process. Despite reports which had indicated earlier that fatty acids were not oxidized by neoplastic tissues (*1–4*), the application of isotope-tracer procedures to this problem revealed that a variety of tumors can oxidize both long-and short-chain fatty acids to CO_2 (*5, 6*). In the meantime, studies of other investigators (*7–10*) also led to the general conclusion that tumor cells utilize fatty acids for respiration.

When fatty acids, *e.g.*, palmitate-1-C^{14}, are fed to mice, they are incorporated into the tumor lipides; by subsequent dissection and aerobic incubation of such labeled tumors, it was found, from the degree of incorporation of C^{14} into the respiratory CO_2, that these fatty acids contribute substantially to the total respiration (*11*). These findings provided additional evidence for a role of fatty acids in tumor respiration, and further suggested that fatty acids may constitute the main fuel for the endogenous respiration of tumor cells. However, they leave unsettled the question of the extent of fatty-acid oxidation when other substrates,

[1] Received for publication August 6, 1959.

[2] This work was aided by grants from the National Cancer Institute, National Institutes of Health, Public Health Service, and the American Cancer Society, Inc.

e.g., glucose, are available. More recently (*12*) we found that the oxidation of endogenous fatty acids in the Ehrlich ascites tumor was inhibited by glucose to the extent of about 70 percent, glucose acting as a preferential substrate for CO_2 formation.

In the present study, the effect of glucose, as well as of other sugars and intermediates, has been extended to the oxidation of exogenous fatty acids by Ehrlich ascites tumor cells. To avoid possible misleading inhibitory effects of free fatty acids, and to simulate physiological conditions as closely as possible, the labeled substrate was used in the form of lipides of blood plasma. The palmitate-labeled plasma was prepared by prior feeding of palmitate-1-C^{14} to mice or rats, followed by isolation of the blood plasma after exsanguination.

Recent studies point to a fatty-acid serum-albumin complex as the transport form of fatty acids—the so-called UFA (*13*) or NEFA (*14*) fraction. It was deemed desirable to compare the oxidation of a serum protein-palmitate complex, as well as of free palmitate, with that of the labeled plasma, and experiments with these substrates are also described.

METHODS

All the experiments were carried out with suspensions of the Lettré strain of the Ehrlich ascites tumor, maintained in mice of the Swiss strain (*12*, *15*). Procedures for isolation, washing, and incubation of cells, and collection and isolation of CO_2, the composition of the medium, and formulas used in calculations are given in previous papers (*12*, *15*). In some instances, where CO_2 isolation was facilitated by addition of carrier carbonate, appropriate corrections were applied. The palmitic acid-1-C^{14} was obtained from the Volk Radiochemical Company on allocation by the United States Atomic Energy Commission, and was appropriately diluted with nonlabeled palmitic acid. This and other chemicals were high grade commercial reagents.

Preparation of labeled plasma.—Approximately 3 mg. (50 μc.) of palmitic acid-1-C^{14} was neutralized with the theoretical quantity of NaOH, and the colloidal dispersion made to a volume of 10 ml. with the aid of a trace of an emulsifying agent, Emulsone B, obtained from the Emulsol Corporation. Normal female mice were given 0.2 ml. of this solution by stomach tube, after which they were killed by cervical fracture, and as much blood as possible was drawn from the heart into a heparinized syringe. This was centrifuged 60 minutes at 3000 rpm and the plasma drawn off and used directly without further treatment. The peak radioactivity of the plasma lipides was reached between 2 and 3 hours, at which time essentially all the radioactivity of the plasma was ether-extractable. Obtainment of larger amounts of labeled plasma was facilitated by the use of rats instead of mice. Intragastric injection of 2 ml. of the palmitate solution in a 300-gm. rat, followed by the removal of blood after 1 hour, yielded approximately 4 ml. of plasma with a fatty-acid specific activity

of about 500 cpm. Rat plasma as a substrate yielded results identical with those of mouse plasma, and all the experiments were conducted with rat plasma. No fractionation of the plasma lipides was attempted, and it is assumed that the labeled palmitate is distributed among all the various fatty-acid-containing lipides. Total activities of the labeled plasma were determined by oxidation of 0.2-ml. samples with chromic acid (*16*).

Preparation of serum protein-palmitate complex.—The method is essentially that of Bragdon and Gordon (*17*). Blood was drawn from the hearts of anesthetized rats, allowed to clot in an ice bath, and centrifuged. The serum was placed in a cellophane bag and dialyzed at + 5° C. for 18 to 20 hours against 1 liter of Krebs-Ringer phosphate solution of *p*H 7.6. The dialysis medium was replaced 3 times during the 18 to 20 hours. One ml. of 0.005 M solution of sodium palmitate-1-C^{14} was placed in a 25-ml. Erlenmeyer flask and evaporated to dryness on a steam bath; 0.3 ml. of water was added to the residue and the flask was gently heated to put the salt in solution. Three ml. of serum was then quickly added, yielding a clear-to-slightly opalescent solution, which can be kept for at least 12 days in the frozen state without loss in stability.

RESULTS

The experiment shown in table 1 revealed that the addition of rat plasma, in amounts ranging from 0.25 to 1 ml., to ascites cell suspensions had no effect on total oxygen uptake or CO_2 output; the values observed agree closely with those obtained with the same cells in a wholly saline medium (*12*, *15*). As anticipated, the zero time control value indicated the presence of small amounts of radioactive CO_2 in the plasma; this was subsequently minimized to negligible quantities by alternately submitting the freshly drawn plasma to unlabeled CO_2 and reduced pressure. The data in column 5 show that radioactivity in the CO_2 increased with increasing plasma in the medium, though not proportionately.

In a time study of plasma palmitate oxidation shown in table 2, there was observed, over periods ranging from 30 to 240 minutes, an essentially

TABLE 1.—Oxidation of labeled plasma palmitate by Ehrlich ascites tumor cells

Cells equivalent to 377 mg. dry weight were suspended in 12 ml. phosphate saline and incubated 2 hours at 37.6° with indicated volume of labeled rat plasma.

Plasma added (*ml.*)	O_2 uptake (*μmoles*)	Respiratory CO_2		
		Amount (*μmoles*)	Specific activity (*cpm*)	Total activity* (*cpm*)
0.25	279	220	908	207,800
0.50	275	218	1307	284,900
1.00	279	220	2228	490,000
1.00†	—	53	698	37,000

*Total activity = specific activity × μmoles CO_2.
†Zero time control.

linear oxygen uptake accompanied by a continuous though slowly declining rate of conversion of fatty-acid carbon to CO_2. It was surprising to discover that the radioactivity in the respiratory CO_2 accounted for almost two thirds of the total plasma radioactivity after 4 hours' incubation.

TABLE 2.—Rate of plasma palmitate oxidation in Ehrlich ascites tumor cells

Cells equivalent to 277 mg. dry weight were incubated in 12 ml. of phosphate saline containing 0.5. ml. of labeled rat plasma for the periods indicated. Total activity of added plasma was 477,000 cpm.

Time (*minutes*)	O_2 uptake (*μmoles*)	Respiratory CO_2	
		Total activity ($cpm \times 10^{-6}$)	Total activity (*percent of added plasma*)
30	77	32. 4	6. 8
60	154	81. 5	17. 1
90	251	122	25. 6
120	309	184	38. 6
180	469	262	54. 9
240	618	308	64. 6

Effect of Glucose on Palmitate Oxidation

With a uniform dosage of 0.5 ml. of labeled plasma and a half-hour incubation period, table 3 shows that the progressive inhibition of oxygen uptake caused by glucose addition was accompanied by an even more marked inhibition of oxidation of labeled plasma palmitate. For example, at 0.001 M glucose, oxygen uptake was unchanged, but palmitate oxidation was depressed 44 percent; at 0.005 M the respective inhibitions were 19 and 73 percent; at 0.01 M they were 30 and 80 percent, and at 0.025 M they were 48 and 83 percent. These effects of glucose on the exogenous

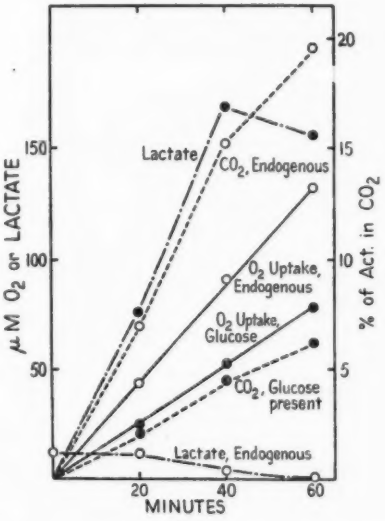
TABLE 3.—Effect of glucose on oxidation of labeled plasma palmitate

Cells equivalent to 178 mg. dry weight were suspended in 12 ml. of phosphate saline containing 0.5 ml. of labeled rat plasma and glucose as indicated, and were incubated for 30 minutes at 37.6°. Total activity of plasma was 111,000 cpm.

Glucose (*molar*)	O_2 uptake (*μmoles*)	Lactate (*μmoles*)	Respiratory CO_2 total activity (*percent*)
None	55	15. 2	10. 2
0. 001	57. 3	43. 7	5. 68
0. 005	44. 4	85. 0	2. 78
0. 01	38. 3	86. 2	2. 01
0. 025	28. 5	78. 5	1. 70

plasma palmitate are similar in magnitude to those observed previously on the oxidation of endogenous palmitate previously incorporated in cell lipides (*12*). Data in column 3 disclose that glycolysis was near maximal at 0.005 M.

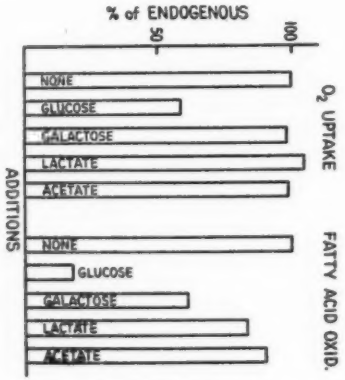
To find out how the oxidation of palmitate is affected by glucose over successive time periods, the experiment shown in text-figure 1 was conducted. During the course of 60 minutes' incubation, oxygen uptake was linear in the presence and absence of glucose, with a constant Crabtree effect of 42 percent inhibition in oxygen uptake; accompanying this was a relatively constant inhibition of 68 to 72 percent in fatty-acid oxidation. With glucose present, a peak in lactic-acid production occurred at 40 minutes in this experiment, at which time 89 of the 132 μmoles of glucose was used up and 168 μmoles of lactate was present, corresponding to 87 percent of the theoretical yield after correcting for the small quantity of endogenous lactate originally present.



TEXT-FIGURE 1.—Time course of palmitate oxidation in the presence or absence of glucose. Cells equivalent to 248 mg. dry weight were incubated for the indicated periods at 37.6° in 12 ml. of a phosphate saline medium, containing 1 ml. labeled plasma. Total activity 290,000 cpm per ml. plasma. Glucose, when present, was at a final concentration of 0.01 M. Experiments without glucose are labeled "endogenous."

Data on the effects of galactose, lactate, and acetate on plasma palmitate oxidation are compared with those of glucose in text-figure 2. In contrast with glucose, galactose exhibited only an extremely low lactate formation (not shown) and, over a range of concentration from zero to 0.025 M, displayed no Crabtree effect. Neither lactate nor acetate significantly affected oxygen uptake. The maximum inhibitory effects

on palmitate oxidation were also low for all 3 substances: 39 percent with galactose, 17 percent with lactate, and 10 percent with acetate. Thus, neither the Crabtree effect nor the associated large depression in palmitate oxidation is shared by galactose or such intermediates of glucose catabolism as lactate or acetate, though all do cause small-to-moderate inhibition of palmitate oxidation. This minor effect of lactate on exogenous palmitate stands in marked contrast with its great effect on the oxidation of endogenous palmitate (*12*).



TEXT-FIGURE 2.—Effects of glucose, galactose, lactate, and acetate, 0.02 M final concentration, on plasma palmitate oxidation. Experiments carried out with the equivalent of approximately 200 mg. dry weight of cells suspended in 12 ml. of a phosphate saline medium containing 0.5 ml. of labeled plasma. Incubation conducted 2 hours in air at 37.6°.

Oxidation of Palmitate as Free Carboxylate Ion and in Serum-Protein Complex

In view of the powerful inhibitory effects of surface-active materials such as the fatty acids, we anticipated that palmitate in the form of the free carboxylate ion might be inhibitory to respiration of the ascites tumor cells. To our surprise, however, no inhibitory effects were observed as long as the palmitate concentration was held at 0.0005 M or below. Above this concentration inhibition was often, though not always, seen, but oxidation was optimal at 0.00025 to 0.0005 M. In recent years a complex of fatty acids with serum albumin has been suggested as a transport form of fatty acids in the plasma, its high turnover rate indicating that this is the form in which fatty acids enter the cells for catabolism (*13*, *14*). Experiments with palmitate-1-C^{14}, in a complex with dialyzed serum protein, revealed that palmitate in this form is also readily oxidized by intact ascites tumor cells, possibly somewhat more readily than is free palmitate. An experiment, in which oxidation of palmitate in the free and protein-bound form was compared in the same cell suspension, is shown in table 4. Oxygen uptake was somewhat in-

creased by palmitate in either form, and CO_2 production from palmitate-1-C^{14} increased with increasing palmitate in either form. It appears from the data of table 4 that protein-bound palmitate is somewhat more efficiently oxidized than is free palmitate. Fillerup, Migliore, and Mead (*10*) also reported the protein-palmitate complex to be more rapidly oxidized than palmitate as such in Ehrlich ascites tumor cells, but this difference between the two forms is not regarded as significant in the present study, since it has not been observed in all experiments. Comparison of the relative specific activities of the fatty acids with those of the respiratory CO_2 reveals that, at the optimal level, exogenous palmitate oxidation accounts for about 5 to 13 percent of the total respiration, depending on the palmitate concentration. The zero time controls (flasks 8 and 9) show that there was no residual CO_2 activity in the substrate in these preparations.

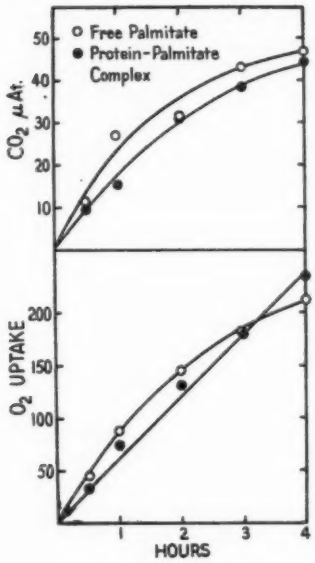
TABLE 4.—Oxidation of palmitate-1-C^{14} as protein-palmitate complex and as free carboxylate ion

Cells equivalent to 126 mg. dry weight were suspended in 12 ml. of fluid and incubated 90 minutes at 37.6°. Flask 1 contained only the phosphate saline medium; flasks 2, 3, and 4 contained, in the 12 ml. total, amounts of 0.8, 2.0, and 4.0 ml., respectively, of a 0.0015 M solution of palmitate-1-C^{14} in dialyzed rat serum; and flasks 5, 6, and 7 contained 0.24, 0.60, and 1.2 ml., respectively, of an 0.005 M solution of sodium palmitate-1-C^{14} in water. Flasks 8 and 9 were set up exactly like 2 and 5, respectively, but were not incubated. Specific activity of palmitate was 7.15×10^5 cpm.

Flask No.	Addition (*molar*)	Oxygen uptake (*μmoles*)	Respiratory CO_2		
			Relative specific activity* (*percent*)	Total activity (*percent*)	Palmitate carbon (*μatoms*)
1	None	81. 9	—	—	—
	Protein-palmitate				
2	0. 0001	81. 3	7. 5	37	7. 17
3	0. 00025	86. 2	12. 3	27	13. 2
4	0. 0005	88. 7	13. 2	17	16. 6
	Palmitate				
5	0. 0001	83. 1	5. 0	27	5. 23
6	0. 00025	87. 5	8. 1	21	8. 15
7	0. 0005	90. 5	12. 3	16. 5	10. 9
	Protein-palmitate†				
8	0. 0001		0. 03		
	Palmitate†				
9	0. 0001		0. 03		

*Relative specific activity = specific activity of $CO_2 \times 100$/specific activity of palmitate.
†Zero time controls.

Text-figure 3 reveals the pattern of oxidation of free palmitate and serum protein-bound palmitate over extended time periods. Over a 4-hour incubation, oxygen uptake was well maintained and the oxidation of both forms occurred at a steadily diminishing rate, which after 4 hours resulted in the conversion to CO_2 of about 50 percent of the added radioactivity. From this and similar experiments we have concluded that palmitate as the free carboxylate ion is oxidized by these ascites tumor cells about as well as palmitate complexed with serum proteins.



TEXT-FIGURE 3.—Time course of palmitate oxidation. The equivalent of 160 mg. dry weight of cells was incubated for the indicated periods in 12 ml. of phosphate saline solution containing palmitate-1-C^{14} in the free or protein-bound form at a final concentration of 0.005 M. Specific activity of substrate was 7750 cpm. O_2 uptake is in μmoles.

Effect of Glucose on Free and Protein-Bound Palmitate Oxidation in Ascites Cells

Table 5 shows the results of an experiment in which glucose was added in concentrations ranging from 0.002 to 0.025 M to the medium containing free palmitate at a concentration of 0.00025 M. Here, the Crabtree effect was not as pronounced as usual, since the oxygen uptake dropped only about 30 percent. However, oxidation of the exogenous palmitate was increasingly inhibited, maximal suppression of palmitate oxidation having occurred at a glucose concentration of 0.005 M. At this concentration, the relative proportion of palmitate carbon in the CO_2 decreased from 13.2 to 5.5 percent, while the total activity in CO_2 declined from 22.1 percent, representing 11.1 μatoms palmitate carbon, to 7.5 percent, representing 3.8 μatoms. In table 6 are the results of a similar experiment with palmitate in the form of the protein complex. Here, too, oxygen uptake was inhibited to a maximum extent of 31 percent, but fatty acid oxidation was cut from a total of 11.1 to 2.9 μatoms palmitate carbon by glucose at 0.005 M, an inhibition of 70 percent. At 0.002 M glucose, where oxygen uptake was inhibited only 9 percent, palmitate oxidation was decreased 38 percent.

TABLE 5.—Effect of glucose on oxidation of palmitate-1-C^{14}

Cells equivalent to 169 mg. dry weight were suspended in 12 ml. of phosphate saline containing glucose in concentrations ranging from 0 to 0.025 M. Incubation was for 30 minutes at 37.6°. Specific activity of palmitate was 18.6×10^3 cpm and final concentration was 0.00025 M.

Glucose (*molar*)	O_2 uptake (*μmoles*)	Respiratory CO_2		
		Relative specific activity (*percent*)	Total activity (*percent*)	Palmitate carbon (*μatoms*)
0	62.6	13.2	22.1	11.1
0.002	55.7	7.2	11.6	5.8
0.005	49.9	4.8	6.9	3.5
0.01	47.0	5.5	7.5	3.8
0.025	46.3	5.5	7.5	3.8

TABLE 6.—Effect of glucose on oxidation of serum protein-bound palmitate-1-C^{14}

Cells equivalent to 147 mg. dry weight were suspended in 12 ml. of of phosphate saline containing glucose in concentrations ranging from 0 to 0.025 M. Incubations were for 30 minutes at 37.6°. Specific activity of palmitate was 18.6×10^3 cpm and final concentration was 0.00025 M.

Glucose (*molar*)	O_2 uptake (*μmoles*)	Respiratory CO_2		
		Relative specific activity (*percent*)	Total activity (*percent*)	Palmitate carbon (*μatoms*)
0	53.6	11.9	19.2	9.7
0.002	48.4	8.3	12.0	6.0
0.005	40.4	4.7	5.7	2.9
0.01	36.3	4.8	6.1	3.1
0.025	37.0	5.0	6.4	3.2

DISCUSSION

The experiments described confirm previous studies in demonstrating a high capability of ascites tumor cells for utilization of fatty acids as a respiratory substrate, and extend the findings previously made on endogenous fatty acids to exogenous palmitate in various forms. Regardless of whether this substrate was in the form of plasma lipides, protein-complex, or free carboxylate ion, its oxidation proceeded steadily for relatively long periods. In these and several other respects our results differed somewhat from those of Fillerup *et al.* (*10*). According to these investigators, free palmitate was oxidized for approximately 1 hour, after which oxidation ceased. On the other hand, albumin complex was oxidized somewhat better, and oxidation continued for at least 90 minutes. Fillerup *et al.* (*10*) also observed respiratory inhibition at palmitate levels far below those that we found to be inhibitory. These

differences could be due to many obvious differences in mouse and tumor strains as well as in experimental methodology.

Extent of palmitate oxidation.—In order to calculate in absolute terms the contribution of exogenous palmitate to the total respiration, it is necessary to know the specific activity of the palmitate undergoing oxidation. This value cannot easily be obtained for the labeled plasma, since the labeled palmitate is distributed among the whole complex of plasma lipides, and we yet do not know the extent to which the individual lipides are absorbed, broken down, and their fatty acids oxidized by individual cell types. However, in those experiments in which palmitate was oxidized as the free or protein-bound form, the specific activity of the palmitate was known, and its conversion to CO_2 was readily computed. In the experiment in table 4, palmitate, depending on its concentration, contributed carbon to the extent of 5 to 13 percent of the total respiratory CO_2 and this accounted, in absolute terms, for the oxidation of from about 5 to 16 μatoms of palmitate carbon. In the experiments of text-figure 3, one can compute that 45 μatoms of palmitate carbon was oxidized in 4 hours, this amount requiring 64 μmoles of oxygen or about one third of the total consumed.

It is important to recognize that these figures do not represent the total fatty-acid contribution, but only that of the exogenous fatty acids. At the same time the exogenous palmitate is undergoing oxidation, part is entering the cell to replace endogenous fatty acids which, as pointed out earlier (*11*), are readily oxidized and may contribute substantially to the total endogenous respiration.

In recent years, the unesterified fatty-acid fraction of the plasma has been implicated as the form in which fatty acids are transported in the blood and absorbed into cells (*10, 13, 14*). Although our studies do not deny a special function of this fraction, they suggest that this is not an obligatory form for cellular catabolism, since (*a*) most of the plasma radioactivity underwent conversion to CO_2 even though only traces could have been present as free fatty acids; (*b*) the rate of the free palmitate oxidation was not markedly less than that of the serum protein-bound palmitate.

Effects of glucose and other intermediates.—With each of the 3 forms of palmitate employed as an exogenous substrate, the effect of glucose was the same, and this, in turn, was the same as its effect on endogenous fatty acids (*12*). In each instance, glucose, when present at concentrations of approximately 0.001 M, *viz.*, below that at which a Crabtree effect is shown, palmitate oxidation was markedly inhibited. From the fact that glucose represents a major source of respiratory CO_2 at this concentration (*12, 15*), glucose evidently replaces a large share of the fatty acids as a respiratory substrate. At glucose concentrations sufficiently high to elicit the Crabtree effect, a further reduction in fatty-acid oxidation ensues, which indicates that the Crabtree effect is exerted, at least in part, on fatty-acid catabolism. As pointed out earlier (*12*), many points of contact exist for interplay between glucose and fatty

acids, but a definitive answer to how the former inhibits the latter must await further study.

It is not yet possible to draw up a balance sheet of respiratory substrates for the Ehrlich ascites tumor; however, a rough estimation may be made from this and previous publications (*12*, *15*). In the presence of adequate glucose, about two thirds of the respiratory CO_2 is derived therefrom, with most of the remainder probably coming from fatty acids, both endogenous and exogenous. Other components of the plasma remain for consideration as possible respiratory substrates, of which the most likely sources are the circulating amino acids. Dickens (*18*) has shown that the ammonia, which accumulates during respiration of tumor slices *in vitro*, may account for as much as half of the endogenous respiration, on the assumption that this is derived from the average amino acids. This suggests not only that amino acids may contribute appreciably to the tumor metabolism but also that there is a rapid protein catabolism in tumors. We have also found this to be true for the Ehrlich ascites tumor cells, and have further found that ammonia production is greatly reduced in the presence of glucose.[3] The contribution of individual amino acids to respiration of ascites cells is another facet of respiratory metabolism requiring further investigation.

REFERENCES

(*1*) Baker, C. G., and Meister, A.: Studies on fatty acid oxidation by normal and neoplastic liver. J. Nat. Cancer Inst. 10: 1191–1198, 1950.

(*2*) Ciaranfi, E.: On the ability of tumor tissue to oxidize fatty acids in vitro. Am. J. Cancer 32: 561–564, 1938.

(*3*) Dickens, F., and Weil-Malherbe, H.: The metabolism of normal and tumor tissue. XX. A comparison of the metabolism of tumors of liver and skin with that of the tissue of origin. Cancer Res. 3: 73–87, 1943.

(*4*) Vestling, C. S., Williams, J. N., Jr., Kaufman, S., Maxwell, R. E., and Quastler, H.: The oxidation of octanoate by liver homogenates from leucemic mice. Cancer Res. 9: 639–644, 1949.

(*5*) Weinhouse, S., Millington, R. H., and Wenner, C. E.: Metabolism of neoplastic tissue. I. The oxidation of carbohydrate and fatty acids in transplanted tumors. Cancer Res. 11: 845–850, 1951.

(*6*) Weinhouse, S., Allen, A., and Millington, R. H.: Metabolism of neoplastic tissue. V. Fatty acid oxidation in slices of transplanted tumors. Cancer Res. 13: 367–371, 1953.

(*7*) Šlechta, L., Jakubovič, A., and Šorm, F.: The nature of the endogenous substrate in Ehrlich ascites tumor. Congr. intern. biochim., Résumés communs., 3e Congr., Brussels 1955, 126. Chem. Abstracts 50: 14095a, 1956.

(*8*) Brown, G. W., Jr., Katz, J., and Chaikoff, I. L.: The oxidative metabolic pattern of mouse hepatoma C_{254} as studied with C^{14}-labeled acetates, propionate, octanoate, and glucose. Cancer Res. 16: 509–519, 1956.

(*9*) Emmelot, P., and Bos, C. J.: Factors influencing the fatty acid oxidation of tumor mitochondria with special reference to changes in spontaneous mouse hepatomas. Experientia 11: 353–354, 1955.

(*10*) Fillerup, D. L., Migliore, J. C., and Mead, J. F.: The uptake of lipo-proteins by ascites tumor cells. J. Biol. Chem. 233: 98–101, 1958.

[3] Lewis, Majane, and Weinhouse: unpublished observations.

(*11*) MEDES, G., PADEN, G., and WEINHOUSE, S.: Metabolism of neoplastic tissue. XI. Absorption and oxidation of dietary fatty acids by implanted tumors. Cancer Res. 17: 127–133, 1957.

(*12*) MEDES, G., and WEINHOUSE, S.: Metabolism of neoplastic tissue. XIII. Substrate competition in fatty acid oxidation in ascites tumor cells. Cancer Res. 18: 352–359, 1958.

(*13*) GORDON, R. S., JR., and CHERKES, A.: Unesterified fatty acid in human blood plasma. J. Clin. Invest. 35: 206–212, 1956.

(*14*) DOLE, V. P.: A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. Clin. Invest. 35: 150–154, 1956.

(*15*) BLOCH-FRANKENTHAL, L., and WEINHOUSE, S.: Metabolism of neoplastic tissue. XII. Effects of glucose concentration on respiration and glycolysis of ascites tumor cells. Cancer Res. 17: 1082–1090, 1957.

(*16*) CALVIN, M.: Isotopic carbon: Techniques in Its Measurement and Chemical Manipulation. New York, John Wiley & Sons, 1949, pp. 9–14.

(*17*) BRAGDON, J. H., and GORDON, R. S., JR.: Tissue distribution of C^{14} after the intravenous injection of labeled chylomicrons and unesterified fatty acids in the rat. J. Clin. Invest. 37: 574–578, 1958.

(*18*) DICKENS, F., and GREVILLE, G. D.: Metabolism of normal and tumor tissue IX. Ammonia and urea formation. Biochem. J. 27: 1123–1133, 1933.

Alternative Pathways of Thymine and Uracil Metabolism in the Liver and Hepatoma [1, 2, 3]

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SUMMARY

By the use of an integrating radiochromatogram scanner, the exchange reactions have been followed between thymine-2-C^{14} or uracil-2-C^{14} and ribonucleosides or deoxynucleosides. The reactions occurred according to the following equations in which thymine is shown as the labeled precursor:

$$T^* + Br \rightleftharpoons T^*r + B \qquad (1)$$

$$T^* + Bdr \rightleftharpoons T^*dr + B \qquad (2).$$

The catalyzing enzymes are found in the supernatant fraction after high-speed centrifugation of the rat liver and Dunning LC 18 hepatoma homogenates. In the normal liver the ratio between the activities for the deoxyriboside-transferring enzyme and the riboside-transferring enzyme is about 3.5, and in the Dunning hepatoma about 0.06. Cytosine riboside and deoxyriboside do not participate in the exchange reactions, while thymine and uracil participate equally well. Exchange reaction (1) is sensitive to arsenate ions, in contrast to reaction (2). The comparative inhibitory effect on the exchange reactions exerted by the three naturally occurring pyrimidines and by 5-fluorouracil has been studied. Rat kidney, brain, and heart do not show any measurable amounts of the deoxyribose-transferring enzyme, nor does tissue from the Novikoff rat hepatoma.—J. Nat. Cancer Inst. 24: 13–29, 1960.

JESSE GREENSTEIN was a pioneer in the development of the concept that cancer metabolism will eventually be described in terms of the individual enzymes that cancer cells contain, and although he was identified with the idea of a "more or less uniform biochemical pattern in tumors," he was fully aware of the many exceptions to this generalization and of the magnitude of the tasks yet to be carried out. Thus in 1956 he stated "that tumors differ in their rates of growth, that certain tumors are more invasive or prone to metastasize than others, that some tumors are affected by X radiation and by administration of certain

[1] Received for publication August 6, 1959.

[2] A preliminary report has been presented earlier (*1*).

[3] Abbreviations used in this paper: B = uracil or thymine, T = thymine, C = cytosine, A = adenine, U = uracil, r = riboside group, dr = deoxyriboside group, PCA = perchloric acid, E = enzyme, P = phosphoryl group. Asterisk is used to denote radioactivity.

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drugs while others are not—all are phenomena which demand differential description in biochemical terms. That this description is so far lacking, no one is more painfully aware than the biochemist" (*2*).

The considerations mentioned by Dr. Greenstein and the immense amount of data that he compiled compel us to realize that tumors must have a combination of uniformity and diversity in their enzyme pattern. To be realistic we must admit that the definitive enzymatic difference between a cancer cell and the normal cell from which it arose is yet to be established *in a single instance*. If this is true, we may as well seek for a cancer cell that differs from a normal prototype as little as possible and try to narrow the spectrum of enzymatic differences that exist in order to decide which differences are definitive.

Studies are now available to suggest that one of the significant differences between cancer tissues and homologous normal tissues may be the loss or the decreased activity of a variety of catabolic enzymes that can be considered competitive with respect to important synthetic pathways (*3*). Previous studies by Canellakis (*4*) and others (*cf. 3*) showed such differences in uracil metabolism and we have noted differences in thymidine metabolism (*5*). The present work has been carried out mainly with the Dunning LC 18 hepatoma, which appears to resemble normal liver more than does the Novikoff hepatoma (*6*). The present report deals with the enzymes that catalyze the reversible reactions converting the free bases, uracil or thymine, to the respective ribosides or deoxyribosides. Reichard and Sköld (*7*) have previously reported on the uracil ribonucleoside phosphorylase in ascites tumor cells and in mammalian liver, but the interpretation of the relative values is difficult and may benefit from comparison with the present data. The reactions under consideration are completely reversible and may be considered as either catabolic or anabolic, depending on the enzymatic milieu in which they are found.

METHODS

Radiochemicals and enzyme material.—Thymine-2-C^{14} and uracil-2-C^{14} were obtained from the New England Nuclear Corporation, Boston, Massachusetts. The 5-fluorouracil was obtained from the Hoffman-LaRoche Company via Dr. Charles Heidelberger. Homogenate or particle-free supernatant fraction was used as enzyme source. The supernatant was prepared by centrifuging a 25 percent homogenate for 60 minutes at 114,000 × g in a Spinco Model L ultracentrifuge. The livers and the other organs used were obtained from male Fischer rats bred at this laboratory. The Dunning hepatoma LC 18 was kept by successive subcutaneous transfers to Fischer rats,[5] and the Novikoff hepatoma by intraperitoneal transfers to Holtzman rats.[6] Partial hepatectomy was done 46 hours before the animals were killed.

[5] Transfers were made by Miss Donna Daenti. We are indebted to Dr. W. F. Dunning for an original pair of Fischer rats and for rats bearing the Dunning LC 18 hepatoma.

[6] The authors are grateful to Dr. Charles Heidelberger for the Novikoff hepatoma material.

Preparation of thymine riboside.—To prove the identity of the thymine-containing substance formed by exchange reaction between thymine and uridine, the compound was purified in the following way: The reaction mixture was precipitated in the cold with perchloric acid (PCA) at a final concentration of 0.2 M. After centrifugation the solution was adjusted to about *p*H 11 with potassium hydroxide (KOH). The potassium perchlorate crystals formed in the cold were spun down and the solution was applied to a Dowex 1, formate column. After washing with distilled water, the mixture of the two pyrimidines and their ribosides was eluted with 0.05 M formic acid. Separation was achieved by using two successive one-dimensional paper chromatographic systems. The first one was the butanol-water-NH_3 system described by Littlefield and Dunn (*8*); the second system was No. 8 according to Fink *et al.* (*9*). The substance was not obtained in crystalline form from aqueous solution.

Assay method.—The incubation mixture for the assays had the composition shown in table 1. A molar ratio of 2 between nucleoside and pyrimi-

TABLE 1.—Composition of incubation mixture*

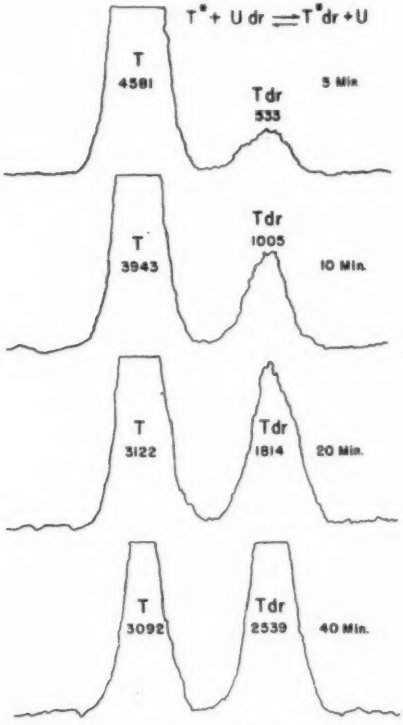
Mixture	Amount (*μmoles*)
Sucrose	438
Tris-buffer, *p*H 8	19. 6
Potassium chloride	8. 6
Magnesium chloride	2. 5
Phosphate buffer, *p*H 8	1. 8
Potassium malate	10. 0
Potassium glutamate	10. 0
Potassium pyruvate	10. 0
Adenosine triphosphate	2. 5
Nucleoside or deoxynucleoside	8. 0
Thymine or uracil (1 or 2 μc., respectively)	4. 0

*Homogenate or supernatant fraction from 250 mg. tissue; final *p*H 7.0; final volume 2.4 ml.

dine base was chosen to promote a rapid transfer of the radioactivity to the nucleoside and thus a more even distribution of the radioactivity during the experiment. Experiments with different substrate levels indicated that the reaction was of the zero order (enzyme dependent) if more than 1 μmole thymine and 2 μmoles deoxynucleoside were used with homogenate from 250 mg. liver. The mixtures were kept in 25-ml. open Erlenmeyer flasks and shaken in a 38° C. thermostated water bath. Aliquots of 400 μl. were taken after 5, 10, 20, and 40 minutes and mixed with the same volume of cold 0.4 M PCA. After centrifugation, the *p*H was adjusted with KOH, with phenol red as an internal indicator. After neutralization, an aliquot of the supernatant fluid (usually 100 μl.) was applied to Whatman #1 paper strips 1 inch wide, and the paper chromatograms were developed with system No. 8 according to Fink *et al.* (*9*) (ethyl-acetate: water:formic acid, 12:7:1). The following phenol red R-values were obtained: thymine 1.10, thymidine 0.77, thymine riboside 0.30, uracil 0.75, deoxyuridine 0.50, and uridine 0.30. The total radioactivity in the aliquots did not show any decrease with time, and the reaction rate was

derived from the radioactivity in the deoxyriboside or riboside spot, expressed as a percentage of the total on the strip. The actual counts for the different radioactive spots were obtained from an integrating radiochromatogram scanner (*10*).[7]

This mixture gives maximal rates of oxidative phosphorylation in whole homogenates, where the combination of malate, glutamate, and pyruvate loads the Krebs cycle most effectively. In high-speed supernatant fractions these substrates are not utilized for adenosine triphosphate (ATP) maintenance. This mixture can be shown to support the conversion of

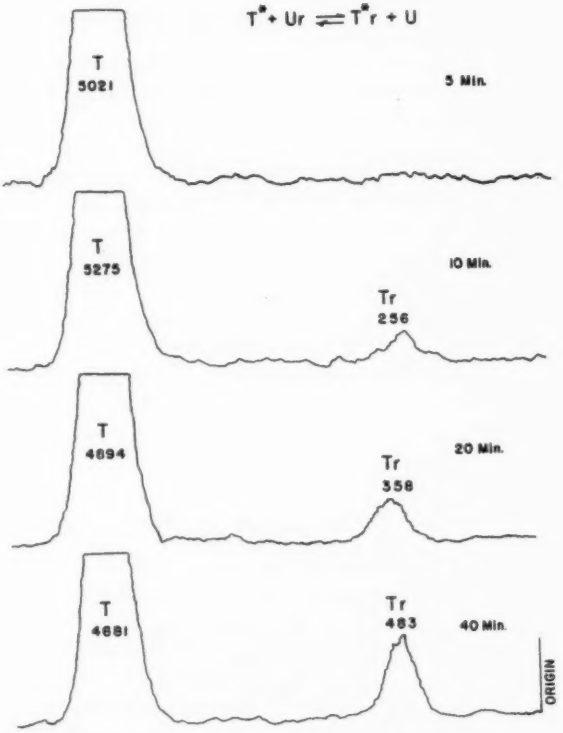


TEXT-FIGURE 1.—The system T* + Udr⇄T*dr + U has been used in this experiment. Four *curves* have been drawn by the inkwriter when the chromatogram strips corresponding to time points 5, 10, 20, and 40 minutes are analyzed by the automatic integrating chromatogram scanner (*10*). Numbers for the *peaks* correspond to the actual values printed by the digital recorder. All final data are based on *fitted curves* or *straight lines* through a series of time points obtained from individual *peaks* such as here shown. In this example, average total count per strip is 5,155 and individual strips show totals of 5,114, 4,948, 4,936, and 5,621. The inkwriter is always set at maximum sensitivity to reveal small *peaks*, and is therefore off-scale for the larger *peaks*, but the digital recorder will integrate the total counts independently of the sensitivity setting that controls the inkwriter (*10*); incubation mixture as in table 1.

[7] The assistance of Dr. Charles Heidelberger, Mrs. Lynn Gilboe, and Mrs. Mary Ward, in all determinations of radioactivity, is gratefully acknowledged.

thymine deoxyriboside (Tdr) to thymidine monophosphate (TMP), thymidine diphosphate (TDP), and thymidine triphosphate (TTP) with some tissues when Tdr with higher specific activity is used, but the formation of these compounds is insignificant under the conditions used here. The reaction mixture is intended to reveal the kinase reactions, if present, and the ATP is not needed for the exchange reactions that predominate.

Evaluation of data.—An exchange reaction that served as a model for the present work has been studied by Duffield and Calvin (*11*), and they



TEXT-FIGURE 2.—*Curves* correspond to those in figure 1, but the system T* + Ur⇄T*r + U has been used instead. Thymine riboside *peak* in these *curves* does not coincide with radioactive thymidine *peak* in text-figure 1.

have given a mathematical interpretation of the data. Their method has been followed for the determination of the half time for the reaction, and from the half time the exchange rate has been calculated by using the following formula:

$$R = k \frac{a \times \ln 2}{3 \times t_{½}}$$

(R = reaction rate, a = μmoles of deoxy- or ribonucleoside, $t_{1/2}$ = half time in min., k = 240 = factor, converting R to μmoles/hr./gm. tissue.) This formula can be derived from an equation given in Duffield and Calvin's paper. Text-figures 1, 2, and 3 give examples of how the values are obtained. The reaction rate is a linear function of the amount of homogenate as verified by text-figure 4.

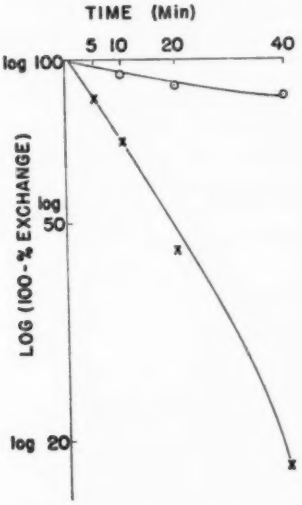
RESULTS

Evidence for Formation of Thymine Riboside

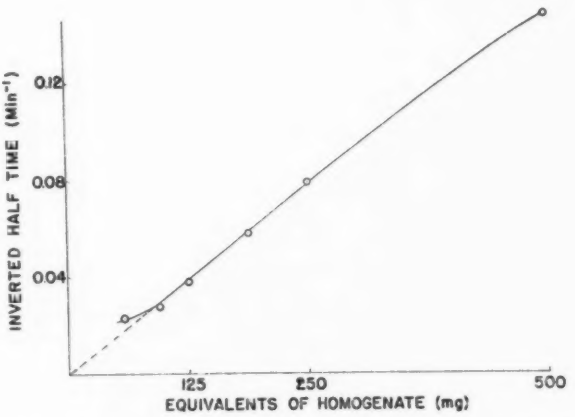
The radioactive compound formed by exchange reaction between labeled thymine and nonlabeled uridine has been purified according to the description given in Methods. The absorption curve between 225 to 290 mμ is very close to the one for thymidine, both at *p*H 1 and *p*H 13. This is in agreement with Littlefield and Dunn who have published almost identical curves for thymidine and thymine riboside (*8*). The compound gave a positive ribose reaction carried out according to Hurlbert *et al.* (*12*). The color developed very slowly, which is explained by the resistance of pyrimidine nucleosides to acid hydrolysis. Text-figure 5 gives the molar extinction values after different periods of heating for the thymine compound compared with those for adenosine, uridine, and thymidine. The curves for the thymine compound and uridine are the most similar. All the ribosides gave color with identical absorption spectrum and a maximum at 660 mμ.

Column Chromatographic Separation of Radioactive Components

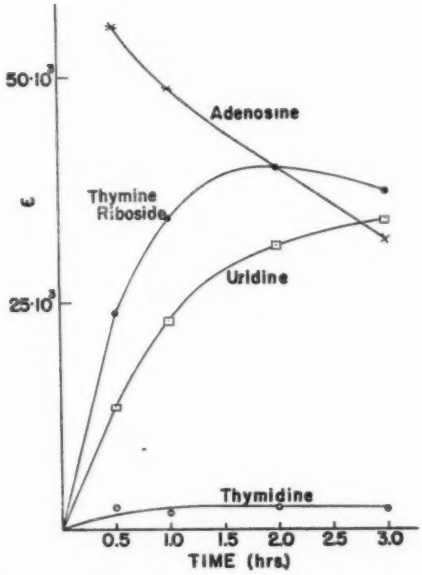
The mathematical calculation of the reaction rate requires that, with the amounts used, the components of the reaction mixture are not metabolized in other directions to a significant extent. This was looked for by determining the total counts per flask, which always kept constant throughout the experiments, and by examining the chromatograms for other radioactive compounds. In addition, an incubation was done, after which the mixture was analyzed with elution chromatography to achieve further separation. After an incubation for 30 minutes with 2 μmoles radioactive thymine (2.4 μc.), 4 μmoles thymidine, and homogenate from 250 mg. liver, the extract was applied to a Dowex 1 column and eluted according to the principles described by Hurlbert *et al.* (*12*). The elution curve is shown in text-figure 6 and it indicates that the main part of the radioactivity is in the combined thymine-thymidine peak. Only about 0.4 percent of the radioactivity is in the next largest peak, which corresponds to 5-carboxyuracil. Reincubation of the material from the 5-carboxyuracil peak indicates that it is not further metabolized under the conditions used.



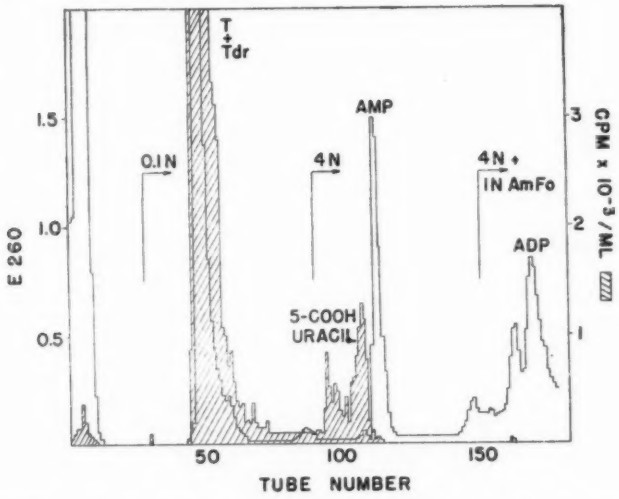
TEXT-FIGURE 3.—Percentage of total radioactivity located in a thymidine or thymine riboside spot has been converted to percent-exchange by dividing by 67/100, which equals the percentage at infinite time with the amounts used, 4 μmoles thymine and 8 μmoles nucleoside or deoxynucleoside; 100 = percent-exchange that can be plotted against time, and from the graph the half time can be obtained.



TEXT-FIGURE 4.—This *curve* shows linearity between inverted (*i.e.*, reciprocal) half time for the reaction, T* + Tdr⇄T*dr + T and amount of enzyme added, expressed as homogenate from a given weight of liver. From the inverted half time the enzyme activity in μmoles per hour per gm. tissue can be obtained by multiplying with a factor.



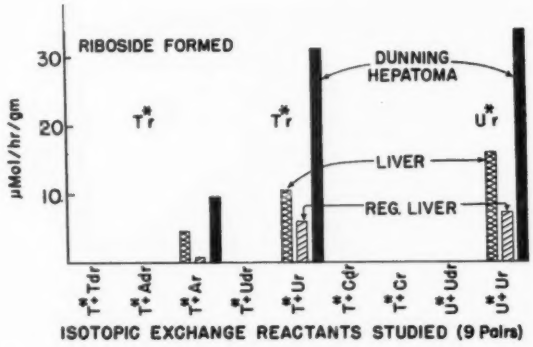
TEXT-FIGURE 5.—Curves show the molar extinction coefficients obtained with the orcinol reaction after different times of heating and with the following substances: adenosine, thymine riboside, uridine, thymidine.



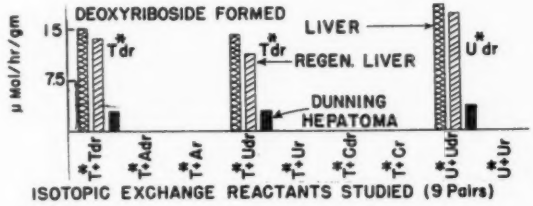
TEXT-FIGURE 6.—The extract from an incubation with radioactive thymine and non-labeled thymidine analyzed by ion-exchange chromatography. Column: Dowex 1, 10×, formate, dimensions 1 × 21 cm. Gradient elution with formic acid and ammonium formate concentrations indicated by *arrows*; volume in mixer 300 ml; volume of fractions 4 ml. Separation was followed by determination of both radioactivity and UV-absorption in the fractions.

Comparison of Enzyme Activity in Homogenate From Liver, Regenerating Liver, and Dunning Hepatoma, With Different Exchange Systems

In this experiment different combinations of substrates were used. Text-figures 7 and 8 give the results from the experiment. Labeled ribonucleosides are formed only when the radioactive base (thymine or uracil) is incubated with an unlabeled ribonucleoside, and labeled deoxyribonucleosides are formed only when unlabeled deoxyribonucleoside is present; thus no detectable reduction or oxidation of the ribo- or deoxy-



TEXT-FIGURE 7.—*Bars* show the ability of homogenates from normal and regenerating liver and the Dunning hepatoma to exchange riboside groups during incubation with different systems. Absence of *bars* indicates the reaction was too small to record on this scale.



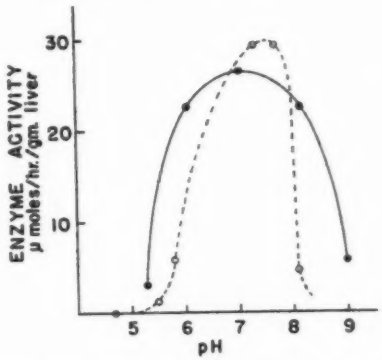
TEXT-FIGURE 8.—Same systems as in text-figure 7 but *bars* show instead the rate with which exchange of deoxyriboside groups occurs; absence of *bars* as in text-figure 7.

nucleosides occurred under these conditions. Cytosinedeoxy- or cytosineribonucleosides did not exchange with thymine. Adenosine and, as has been shown in another experiment, inosine can exchange with a lower rate than uridine, but deoxyadenosine and deoxyinosine do not react at all. The transribosidation activity of the Dunning hepatoma is comparatively much higher than that of normal liver. For transdeoxyribosidation the normal liver tissue shows the highest activity.

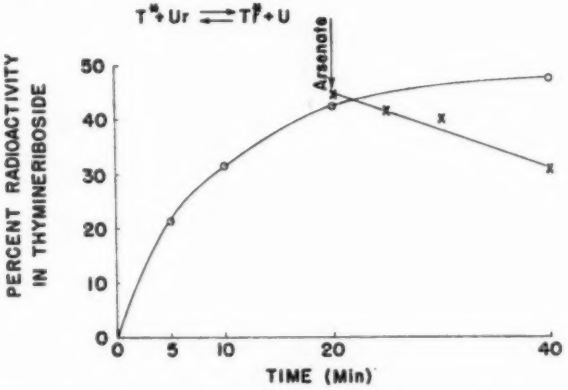
Intracellular Localization, Stability, and *p*H Optimum

Friedkin and Roberts (*13*) found about 40 percent more thymidine phosphorylase in the combined microsomal and soluble fraction than in the whole homogenate. The same is valid also for the riboside- and deoxyriboside-exchanging enzymes in this study. For both systems about 150 percent of the activity of the homogenate was found in the supernatant fraction after high-speed centrifugation.

The activity of the transdeoxyribosidation enzyme in a supernatant fraction was unchanged after 14 days at −10° C. For the transribosidation enzyme a loss of about 30 percent had occurred during the same time. The *p*H curves for the two types of enzyme are shown in text-figure 9.



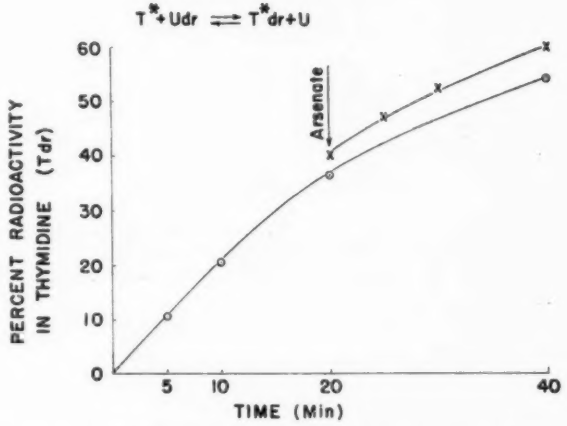
TEXT-FIGURE 9.—The *p*H *curve* for the exchange rate for the reaction T* + Udr⇄T* dr + U (*solid line*) and the reaction T* + Ur⇄T* r + U (*dashed line*). The *p*H deviations obtained by adding potassium hydroxide or hydrochloric acid; the values controlled by glass electrode.



TEXT-FIGURE 10.—Effect of arsenate on the exchange system T* + Ur⇄T*r + U. Arsenate, 120 μmoles, was added after 20-minute incubation, and the effect was followed by samples taken after 20, 25, 30, and 40 minutes. The *curve* that starts from zero time represents the control with no arsenate added.

Effect of Arsenate

To obtain evidence for the existence of ribose-1-phosphate or deoxyribose-1-phosphate as intermediates in the exchange reactions, arsenate was added to the incubation mixture after 20 minutes, in some experiments. The curves in text-figures 10 and 11 are obtained from two such experiments: one with thymine, uridine, and hepatoma supernatant, the other with thymine, deoxyuridine, and liver supernatant. In the former experiment the thymine riboside formed was presumably broken down through depletion of ribose-1-phosphate; in the latter experiment the exchange rate was not altered.



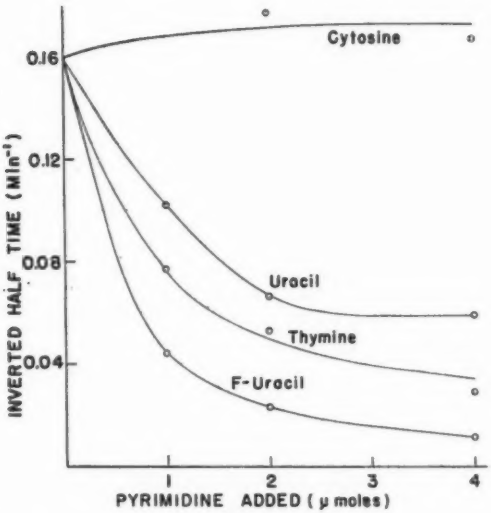
TEXT-FIGURE 11.—Effect of arsenate on the exchange system T* + Udr⇆T*dr + U. Arsenate, 120 μmoles, was added after 20-minute incubation and samples were taken as described in legend of text-figure 10.

Effect of Pyrimidines on Exchange Rate

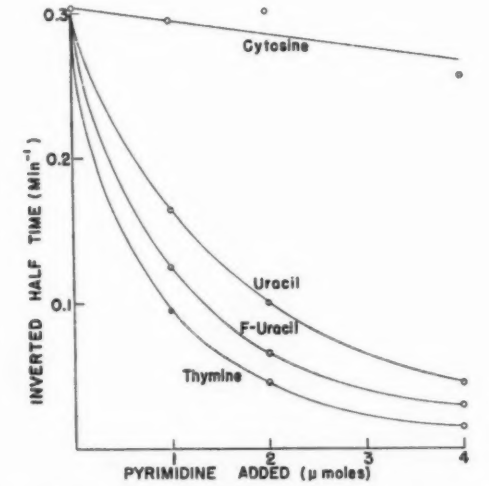
In these experiments a basic system of 1 μmole of uracil-2-C^{14} and 8 μmoles of uridine or deoxyuridine was used. In the incubation with uridine, supernatant from the hepatoma and, in the incubation with deoxyuridine, supernatant from the normal liver were utilized as enzyme source. The effect of addition of 1, 2, or 4 μmoles of the three natural pyrimidines and of 5-fluorouracil was studied, and the results can be seen in text-figures 12 and 13. As expected, cytosine showed only a slight influence on the system. 5-Fluorouracil gave the strongest effect in the riboside-exchanging system, but in the deoxyriboside-exchanging system its effect was intermediate between uracil and thymine.

Enzyme Contents in Different Organs and Hepatoma

The combination thymine-uridine has been chosen to study the riboside-exchanging enzyme, and the combination thymine-deoxyuridine has



TEXT-FIGURE 12.—One, 2, or 4 μmoles of cytosine, uracil, thymine, and 5-fluorouracil were added to the basic system of 1 μmole radioactive uracil and 8 μmoles nonlabeled uridine. *Curves* show the competitive effect exerted by the pryimidines on the exchange system.



TEXT-FIGURE 13.—Experiment similar to that explained in text-figure 12, but the exchange reaction is a transdeoxyribosidation obtained with 1 μmole radioactive uracil and 8 μmoles nonlabeled deoxyuridine.

been selected to study the deoxyriboside-exchanging enzyme in some organs and in two kinds of hepatoma, Dunning and Novikoff. The results of the assays are listed in table 2.

TABLE 2.—Enzyme activities in homogenate (H) or supernatant fraction (S3) from different rat organs and hepatoma—data are on the basis of fresh weight

Experiment	Tissue	(H) or (S3)	Udr system T* + Udr ⇅ T*dr + U (μmole/gm./hr.)	Ur system T* + Ur ⇅ T*r + U (μmole/gm./hr.)	Ratio Udr/Ur system
IX	Liver	H	17	9.9	1.7
XII	"	H	18	4.2	4.2
XII	"	S3	28	6.0	4.6
XIV	"	S3	27	4.5	6.0
XIV	"	S3	24	3.6	6.6
XIV	"	S3	23	5.6	4.2
XX	"	S3	9.3	7.5	1.2
XX	"	S3	9.3	6.4	1.4
XXII	"	S3	8.9	5.6	1.6
Mean	"		18	5.9	3.5
XXII	Liver (newborn rats)	S3	0.5	<0.3	
	" (1-week-old rats)	S3	4.9	1.5	3.3
XI	Dunning hepatoma	H	4.3	34	0.13
XIII	" "	H	2.5	32	0.08
XIII	" "	S3	2.5	52	0.05
XXII	" "	S3	0.5	38	0.01
XXII	" " (intraperitoneally grown)	S3	2.8	81	0.03
Mean	Dunning hepatoma		2.5	47	0.06
XX	Novikoff hepatoma	S3	<0.3	11	
XXII	" "	S3	0.06	7.3	0.008
XX	Kidney	S3	<0.3	5.1	
XX	Brain	S3	<0.3	1.8	
XX	Heart	S3	<0.3	4.5	
XXIII	Spleen	S3	<0.3	3.5	
XXIII	Thymus	S3	<0.3	2.4	

DISCUSSION

With the combination of paper chromatography and the automatic integrating chromatogram scanner (*10*), a simple and nonlaborious method with wide application has been developed. Almost all data in this study have been obtained with this method.

All the experiments indicate that at least two enzymes are involved in the two types of reactions studied, the riboside exchange and the deoxyriboside exchange. This conclusion is based on the distribution in the different organs and hepatoma and the effect of arsenate, *p*H, and fluorouracil.

The experiment with arsenate addition suggests that the riboside-exchange reaction is not a pure exchange reaction but the summation of two phosphorylase reactions. A similar conclusion can be drawn from the work of Ott and Werkman, with enzyme from *Escherichia coli* (*14*). In the deoxyribosidation the question arises whether the active enzyme is identical with the thymidine phosphorylase described by Friedkin and Roberts (*13*, *15*), since arsenate ions do not change the reaction rate. Work by Hoffmann (*16*) shows that a pyrimidine-exchange in extract from *E. coli* is not phosphate ion dependent, though the extract contained phosphorylase activity. The results are best explained by assuming that the same enzyme can act both as a phosphorylase and a

trans-*N*-deoxyribosylase, in the latter without liberation of deoxyribose-1-phosphate. If the enzyme is assumed to catalyze the following reactions:

$$\text{Bdr} + \text{E} \rightleftharpoons \text{EBdr} \quad (1)$$
$$\text{EBdr} \rightleftharpoons \text{Edr} + \text{B} \quad (2)$$
$$\text{Edr} + \text{P} \rightleftharpoons \text{E} + \text{drP} \quad (3)$$
$$\text{Bdr} + \text{P} \rightleftharpoons \text{B} + \text{drP}$$

it will through reactions (1) to (3) function as a phosphorylase and through reactions (1) and (2) function as a trans-*N*-deoxyribosylase. If reactions (1) and (2) are more rapid than (3), arsenate will not exert any marked effect on the exchange. Further conclusions on the mechanism of action for the two enzymes require experiments with ribose-1-phosphate, deoxyribose-1-phosphate, and purification of these enzymes.

Several earlier reports show that mammalian extracts are incapable of ribo- or deoxyribonucleoside phosphorylase activities with cytidine or deoxycytidine as substrate (*15*, *17*, *18*), and these reports are in line with our findings. According to Rousch and Betz (*19*), this metabolically important limitation is not valid for deoxyribose transfer reactions in *Lactobacillus helveticus*, and they found no deoxyribose transfer in the rat liver, but they assayed for the enzyme with only deoxyinosine and the bases adenine and guanine as substrates. Their results are thus similar to our finding that thymine does not exchange with deoxyadenosine or deoxyinosine.

Comparison between the different organs and livers from rats of different ages (table 2) seems to indicate that the deoxyribose-transferring enzyme is found exclusively in the liver and tissues derived from the liver. Friedkin and Roberts in their study of thymidine phosphorylase (*13*) assayed for the enzyme in different organs. According to their results, the kidney contained about 17 percent of the activity of the liver. The spleen contained a still lower concentration and the amount in the heart and brain was not measurable.

The test system described in this report may be useful for studying the effects of the halogenated pyrimidine derivatives on these reactions. An inhibitory effect of fluorouracil on pyrimidine nucleoside phosphorylase has been described by Sköld (*20*). It is probably a little misleading to speak of fluorouracil as an inhibitor of these reactions since it appears to act as a competitive substrate. This is brought out in text-figures 12 and 13 in which nonradioactive pyrimidines, with structures identical or sufficiently similar to those of the radioactive precursor, behave alike. The inhibition noted is a result of the ability of the analogues to combine with the enzyme, and the distinction between an inhibitor and a competitive substrate is that the latter is metabolized to a product analogue and is used up during the course of the reaction. Some preliminary experiments with labeled fluorouracil in comparison with labeled thymine have shown that the former reacts 0.58 times as fast in the ribose-transferring system and 1.7 times as fast in the deoxyribose-transferring system.

Further studies will be needed to describe analogues of this type in terms of both Michaelis constants and reaction rates in any given reaction.

From work by Pitot *et al.* (*6*) the Dunning hepatoma seems to have an enzyme pattern with levels that fit in between normal liver and Novikoff hepatoma. This rule appears valid also for the trans-*N*-deoxyribosylase, but not for the ribose-transferring enzyme in this study.

The meaning of the difference in the distribution of the two enzymes between the normal liver and Dunning hepatoma is not obvious at this time. However, if the deoxyribose-exchanging enzyme within the cells acts mainly as a degrading phosphorylase, there would be less breakdown of thymidine in the tumor tissue and more thymidine available for deoxyribonucleic acid (DNA) synthesis via thymidine kinase. Studies on the latter enzyme are in progress because this enzyme would be needed to utilize any thymidine made available by its decreased breakdown. In large invasive cancers the production of Tdr from the thymidylic acid of deoxyribonucleic acid in adjacent normal tissue by the combined action of DNases, diesterases, and thymidylic phosphatases, in the absence of effective conversion of Tdr to thymine, might constitute an effective means for bypassing the conversion of deoxyuridylic acid to thymidylic acid. It is the latter step that is considered a major site of action for the fluorinated pyrimidines (*21*, *22*). In seeking ways to support the action of the latter class of compounds, we need combination therapy with agents that block the utilization of Tdr for DNA synthesis. These agents are apparently available (*23*, *24*).[8]

In seeking an explanation for the *increase* in the ribose-transferring enzyme in the hepatomas relative to the liver, we are at the moment inclined to regard the *thymine* ribosidation as a phenomenon with little physiological significance, because thymine is probably not available to the enzyme *in vivo*, and our attempts to show further metabolism of thymine riboside have been unrewarding. There appears to be a weak phosphorylation by ATP in the presence of the supernatant fraction of the Dunning hepatoma, but the reaction is minor in comparison with Tdr or Ur. No evidence for reduction of the ribose moiety or for phosphorylation beyond the monophosphate has been obtained. The formation of thymine riboside for the rat liver was previously reported by Fink *et al.* (*9*) and by Friedkin and Roberts (*15*).

Littlefield and Dunn (*8*) have found a low percentage of thymine in RNA from *E. coli* and *Aerobacter aerogenes*, and Griffin *et al.* (*25*) have shown that a bacterial polynucleotide phosphorylase can polymerize thymine riboside diphosphate, which was prepared synthetically. These observations indicate that at least in some microorganisms the pathway from thymine riboside phosphate to the diphosphate should be open to a limited extent.

The increase in the ribose-transferring enzyme is probably significant in the utilization of *uracil* for the synthesis of uridine and its subsequent

[8] Experiments by Dr. Charles Heidelberger suggest improved responses, in certain cases, by combination therapy with 5-fluorouracil and 5-iododeoxyuridine.

conversion to ribonucleotides and ribonucleic acid (RNA). This was also the conclusion of Reichard and Sköld who found the enzyme in ascites tumor cells (*7*). The present comparison of the liver and hepatoma cells provides additional documentation for the rationale of tumor chemotherapy with fluorouracil, as described by Harbers *et al.* (*21*), since it shows that in addition to the previously documented decrease in the breakdown of uracil in the hepatoma, as compared with the liver, there is an *increased* conversion to the riboside.

There is thus a strong indication that tumor cells may possess a collection of enzymes that facilitate the reutilization of preformed uracil and thymine and their nucleosides by a combination of decreased breakdown and increased utilization (*3–5*).

No attempt is made to review the growing literature on the conservation of the building blocks for protein and nucleic acid synthesis in tumors in terms of lowered catabolic activity for various intermediates. The tendency of a tumor to avoid the breakdown of free pyrimidines and purines should result in a high utilization of exogenous supplies of these compounds if it were not that the total organism is able to catabolize these compounds effectively in the liver. The important question of whether the ability of the tumor to conserve free purines and pyrimidines and their nucleosides is a purely internal phenomenon or whether it is adapted to the utilization of host building blocks at the local level has apparently not been attacked.

Further studies will be needed to discover whether the intracellular localization of the enzymes is such as to facilitate the utilization of host nucleic acids by the tumors in local sites. A high concentration of thymidylic and uridylic phosphomonoesterases in the tumor-cell membrane might be expected to expedite such conversions, and we are accordingly led to place increased emphasis on cytochemical and cell-fractionation techniques, and to use whole homogenates only as one small part of the over-all approach.

Jesse Greenstein felt that "the host-tumor relationship is the key to the cancer problem" (*2*), and in pursuing the mechanism by which normal tissues regulate their reutilization of preformed building blocks, while tumors seemingly revise their enzyme pattern so as to facilitate the capture of host building blocks, we may usefully follow some of the many leads that Dr. Greenstein helped to provide.

REFERENCES

(*1*) Potter, V. R., and de Verdier, C.-H.: Alternative pathways of thymine metabolism. (Abstract.) Proc. Am. Assoc. Cancer Res. 3: 53, 1959.

(2) Greenstein, J. P.: Some biochemical characteristics of morphologically separable cancers. Cancer Res. 16: 641–653, 1956.

(3) Potter, V. R.: The biochemical approach to the cancer problem. Fed. Proc. 17: 691–697, 1958.

(*4*) Canellakis, E. S.: Pyrimidine metabolism. III. The interaction of the catabolic and anabolic pathways of uracil metabolism. J. Biol. Chem. 227: 701–709, 1957.

(5) Potter, V. R., Brumm, A. F., and Bollum, F. J.: Metabolism of thymidine in relation to DNA synthesis in rat liver and Novikoff hepatoma. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 336, 1958.

(6) Pitot, H. C., Fohn, C. H., Clark, W. H., Jr., and Farber, E.: A comparative biochemical and morphological study of some transplanted and primary rat hepatomas. (Abstract.) Proc. Am. Assoc. Cancer Res. 3: 52, 1959.

(7) Reichard, P., and Sköld, O.: Enzymes of uracil metabolism in the Ehrlich ascites tumour and mammalian liver. Biochem. et biophys. acta 28: 376–385, 1958.

(8) Littlefield, J. W., and Dunn, D. B.: The occurrence and distribution of thymine and three methylated-adenine bases in ribonucleic acids from several sources. Biochem. J. 70: 642–651, 1958.

(9) Fink, K., Cline, R. E., Henderson, R. B., and Fink, R. M.: Metabolism of thymine (methyl-C^{14} or -2-C^{14}) by rat liver *in vitro*. J. Biol. Chem. 221: 425–433, 1956.

(10) Ludwig, H., Potter, V. R., Heidelberger, C., and de Verdier, C.-H.: Automatic direct quantitation of radioactivity on paper chromatograms. Biochem. et biophys. acta. In press.

(11) Duffield, R. B., and Calvin, M.: The stability of chelate compounds. III. Exchange reactions of copper chelate compounds. J. Am. Chem. Soc. 68: 557–561, 1946.

(12) Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V.: Nucleotide metabolism. II. Chromatographic separation of acid-soluble nucleotides. J. Biol. Chem. 209: 23–39, 1954.

(13) Friedkin, M., and Roberts, D.: The enzymatic synthesis of nucleosides. I. Thymidine phosphorylase in mammalian tissue. J. Biol. Chem. 207: 245–256, 1954.

(14) Ott, J. L., and Werkman, C. H.: Coupled nucleoside phosphorylase reactions in *Escherichia coli*. Arch. Biochem. 69: 264–276, 1957.

(15) Friedkin, M., and Roberts, D.: The enzymatic synthesis of nucleosides. II. Thymidine and related pyrimidine nucleosides. J. Biol. Chem. 207: 257–266, 1954.

(16) Hoffmann, C. E.: Desoxyribose transfer by nucleosidases of *Escherichia coli*. (Abstract.) Fed. Proc. 11: 231, 1952.

(17) Cardini, C. E., Paladini, A. C., Caputto, R., and Leloir, L. F.: Liver uridine phosphorylase. Acta physiol. Latinoamericana 1: 57–63, 1950.

(18) Boxer, G. E., and Shonk, C. E.: Deoxyribose-5-phosphate metabolism by normal liver and malignant hepatoma. J. Biol. Chem. 233: 535–540, 1958.

(19) Rousch, A. H., and Betz, R. F.: Purification and properties of trans-N-deoxyribosylase. J. Biol. Chem. 233: 261–266, 1958.

(20) Sköld, O.: Enzymic ribosidation and ribotidation of 5-fluorouracil by extracts of the Ehrlich-ascites tumor. Biochem. et biophys. acta 29: 651, 1958.

(21) Harbers, E., Chaudhuri, N. K., and Heidelberger, C.: Studies on fluorinated pyrimidines. VIII. Further biochemical and metabolic investigations. J. Biol. Chem. 234: 1255–1262, 1959.

(22) Chaudhuri, N. K., Mukherjee, K. L., and Heidelberger, C.: Studies on fluorinated pyrimidines. VII. The degradative pathway. Biochem. Pharmacol. 1: 328–341, 1959.

(23) Prusoff, W. H.: Synthesis and biological activities of iododeoxyuridine, an analog of thymidine. Biochem. et biophys. acta 32: 295–296, 1959.

(24) ———: Incorporation of iododeoxyuridine, an analog of thymidine, into mammalian deoxyribonucleic acid. (Abstract.) Fed. Proc. 18: 305, 1959.

(25) Griffin, B. E., Todd, A., and Rich, A.: A synthesis of ribothymidine-5′-pyrophosphate and its conversion to polyribothymidylic acid. Proc. Nat. Acad. Sc. 44: 1123–1128, 1958.

Recent Investigations of L- and D-Amino Acid Oxidases[1, 2, 3]

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SUMMARY

Recent research on the flavoprotein amino acid oxidases is reviewed, and several problems relating to the physiological significance of the oxidases and the mechanism of the enzymatic reaction are discussed. The available evidence appears to exclude formation of an α,β-unsaturated intermediate in the reaction, and suggests participation of an imino acid intermediate. Synthetic Δ^1-pyrroline-2-carboxylic acid, the presumed intermediate in the oxidation of proline, was available for direct examination of its possible participation in the amino acid oxidase reaction. Thus, enzymatic synthesis of D-proline from Δ^1-pyrroline-2-carboxylic acid was demonstrated. Synthesis of other D-amino acids from the corresponding α-keto acids and ammonia was also catalyzed by D-amino acid oxidase. Reversibility of the reaction catalyzed by L-amino acid oxidase was also demonstrated. These studies support the belief that an imino acid intermediate is involved in the amino acid oxidation reaction, and make possible new approaches to the study of the mechanism of the enzymatic reactions. Studies on the inhibition of amino acid oxidase by high concentrations of substrate and the effect of oxygen tension are described; a tentative explanation for the results is proposed in which the formation of a semiquinone intermediate is considered. Reactions catalyzed by D-amino acid oxidase involving D-allohydroxyproline and D-pipecolic acid are described. The nonenzymatic formation of pyrrole-2-carboxylic acid from Δ^1-pyrroline-4-hydroxy-2-carboxylic acid (formed enzymatically from D-allohydroxyproline) has provided a highly sensitive method for determining D-amino acid oxidase activity. Crystalline L-amino acid oxidase has been prepared in this laboratory from rattlesnake venom, which contained three separable L-amino acid oxidases. Significant differences between the L-amino acid oxidases of rattlesnake and moccasin venoms are discussed. The physiological significance of D-amino acid oxidase and several possible explanations for its presence in mammalian tissues are considered. It was

[1] Received for publication September 3, 1959.

[2] Supported by the National Institutes of Health, Public Health Service, and the National Science Foundation.

[3] *Chemical Abstracts'* nomenclature for compounds in this paper: Δ^1-pyrroline-2-carboxylic acid = 1-pyrroline-2-carboxylic acid; Δ^1-pyrroline-4-hydroxy-2-carboxylic acid = 4-hydroxy-1-pyrroline-2-carboxylic acid; α-keto-β-methylvaleric acid = 3-methyl-2-oxovaleric acid; β-phenylserine = 3-phenylserine; α-ketoisocaproic acid = 4-methyl-2-oxovaleric acid; glycyldehydrophenylalanine = 2-(2-amino-acetamido)-cinnamic acid; α-keto-δ-aminovaleric acid = 5-amino-2-oxovaleric acid; δ-*N*-carbobenzoxy-α,δ-diaminovaleric acid = N^{δ}-benzyloxycarbonylornithine; α-aminoadipic-δ-semialdehyde = 5-formylnorvaline; Δ^1-piperidine-6-carboxylic acid = 2,3,4,5-tetrahydropicolinic acid; hydroxyproline = 4-hydroxyproline; Δ^1-piperidine-2-carboxylic acid = 3,4,5,6-tetrahydropicolinic acid.

[4] Predoctoral Research Fellow, National Cancer Institute, National Institutes of Health, Public Health Service.

found that the kidneys of germ-free mice exhibit D-amino acid oxidase activity comparable to that of the kidneys of mice raised under nonsterile conditions. These studies appear to indicate that D-amino acid oxidase activity does not develop adaptively to D-amino acids formed by the bacterial flora.—J. Nat. Cancer Inst. 24: 31–49, 1960.

AMINO ACID oxidases have served biological science for many years as unique biochemical tools. Shortly after its discovery, D-amino acid oxidase was used by Warburg and Christian in their fundamental studies on flavine adenine dinucleotide [FAD, (*1*, *2*)]. In his classical work on amino acid resolution, Greenstein utilized the extraordinarily high optical specificity of the D- and L-amino acid oxidases to show that amino acids obtained by the acylase and amidase methods exhibited optical purity greater than 99.9 percent and in certain cases greater than 99.99 percent (*3*, *4*). Other applications of the amino acid oxidases in the preparation and study of amino acid isomers may be found in Greenstein's work and in other investigations, such as in the preparation of α-keto acids and other compounds and in studies of racemases.

Although the amino acid oxidases have been useful to the chemist and the biochemist, the physiological functions of these enzymes are not yet clearly defined. Thus not only are we uncertain as to how the L-amino acid oxidase of the rattlesnake venom serves this animal, but the function of L-amino acid oxidase in mammalian tissues is not yet clear. The low L-amino acid oxidase activity of various mammalian tissues gives indirect support to the belief that deamination of amino acids takes place mainly by coupled action of glutamic dehydrogenase and glutamic transaminase, as suggested originally by Braunstein and Bychkov (*5*). It is curious that, in contrast to the relatively feeble L-amino acid oxidase activity of mammalian tissues, mammalian liver and kidney contain considerable D-amino acid oxidase activity. Although D-amino acids have been found in the cell walls of certain bacteria, in other products of microorganisms, and elsewhere, there is at this time no good evidence for the occurrence of D-amino acids in mammalian tissues, either in peptide linkage or in other forms. Whether or not D-amino acid oxidase carries out a function in the mammal, its widespread occurrence in the organisms of this class remains to be explained.

What are the structural features of the L-amino acid oxidase of rattlesnake venom and of the D-amino acid oxidase of hog kidney that insure the absolute specificity of one enzyme for L-amino acids and of the other for D-amino acids? It appears that both enzymes are themselves composed of L-amino acids, and that FAD is the prosthetic group for both enzymes. The subtleties of the protein structures and the mechanisms involved in the attachments of the prosthetic group and the substrates, which are responsible for this remarkable optical specificity, require investigation. An associated problem is elucidation of the mechanism of the amino acid oxidase reaction. Although the over-all reaction catalyzed has long been

known, and appears to be the same for both D- and L-amino acid oxidases, we have little information concerning the details of the reaction mechanism and the intermediates involved.

In this paper, the authors have attempted to review some recent research on the flavoprotein amino acid oxidases, to consider the questions raised, and to summarize some information obtained in this laboratory concerning these enzymes.

STUDIES ON PURIFICATION AND NATURE OF FLAVOPROTEIN AMINO ACID OXIDASES

The oxidative deamination of L- and D-amino acids by crude preparations of tissues and microorganisms was demonstrated in a number of early studies (*6–8*). Subsequently, D-amino acid oxidase was isolated from sheep kidney by Negelein and Brömel (*9*); it was shown that the enzyme contained FAD as the prosthetic group (*1*, *2*). An alternative procedure for the preparation of this enzyme, in which the protective effect of *m*-toluic acid on the enzyme was utilized, has been described (*10*). Neither of these procedures yields an enzyme of much greater than 70 percent purity.[5] Negelein and Brömel estimated that approximately 100,000 grams of their purified sheep-kidney D-amino acid oxidase contained 1 mole of FAD.

We have hydrolyzed preparations of purified sheep-kidney D-amino acid oxidase with 6 N hydrochloric acid for 18 hours at 108° C., and have incubated such hydrolysates with D-amino acid oxidase. Although evidence of D-amino acids in these hydrolysates was obtained, the quantities of D-amino acids were approximately the same as those found in hydrolysates of crystalline bovine-serum albumin prepared in the same manner; in neither case was the total quantity of D-amino acids, as determined with the oxidase, greater than 1 percent of the total amino acids present. Although these results do not exclude the possibility that some D-amino acids occur in these proteins, they indicate that D-amino acid oxidase, like other proteins, is chiefly if not entirely composed of L-amino acids. The presence of small amounts of D-amino acids in protein hydrolysates may be due to racemization during hydrolysis, or conceivably to the presence of D-amino acids in the original protein.

D-Amino acid oxidase activity has been found also in octopus hepatopancreas (*12*), and in several molds and bacteria (*13–16*, *8*). The octopus hepatopancreas enzyme, in contrast to the mammalian kidney D-amino acid oxidase, oxidizes D-glutamic acid and D-aspartic acid. Evidence has been found for a specific enzyme in rabbit liver and kidney that oxidizes D-aspartic acid and to some extent D-glutamic acid (*17*, *18*). Unfortunately, highly purified preparations of these D-amino acid oxidases are

[5] Kubo *et al.* (*11*) have reported isolation of crystalline D-amino acid oxidase from pig kidney; however, essential information concerning characterization of the enzyme, *e.g.*, specific activity, electrophoresis, and ultracentrifugation, was not given, and complete experimental details on the method of isolation were not reported.

not yet available, though some progress has been made recently in purifying the octopus hepatopancreas enzyme (*19*).

L-Amino acid oxidase activity has been found in the rat kidney (*20, 21*), *Neurospora crassa* (*22, 23*), *Proteus vulgaris* (*24*), other microorganisms (*13, 25, 22*), and venoms of various snakes (*26*). Since the reports from Green's laboratory (*20, 21*), no further work apparently has been published on the purified L-amino acid oxidase of rat kidney. However, the original studies are of interest in that a very highly purified enzyme preparation was obtained that possessed flavine mononucleotide as the prosthetic group. The electrophoretically homogeneous enzyme consisted of two forms with molecular weights of 138,000 and 550,000, respectively. The enzyme also oxidized a number of α-hydroxy acids, and L-α-hydroxy acids were more rapidly oxidized than L-α-amino acids. The enzyme exhibited a low turnover number, and apparently was difficult to isolate from kidneys of other species. It is conceivable that the isolated enzyme represents a portion of a more complex integrated amino acid oxidase system, perhaps associated with the mitochondria. The L-amino acid oxidase of *P. vulgaris* studied by Stumpf and Green (*24*) was found to be associated with particles that sedimented at 3,000 $\times$ g. Boulanger and Osteux have discovered an L-amino acid oxidase in turkey liver, which appears to be specific for the basic amino acids; they have shown that the enzyme attacks the α-amino groups of lysine and ornithine (*27–29*).

In 1944, Zeller and Maritz (*30*) made the interesting observation that the venom of a number of snakes contained large amounts of L-amino acid oxidase. The enzyme was also found in the tissues of the snakes. Zeller showed that the yellow color of venom was due partly to its riboflavine content and that the intensity of color was roughly parallel to the enzyme activity (*26*). Singer and Kearney (*31, 32*) succeeded in isolating the enzyme in a highly purified state from moccasin, *Agkistrodon piscivorus*, venom and demonstrated that FAD was the prosthetic group. Moccasin venom and the venom of several other snakes, *e.g.*, rattlesnake venom, contain as much as 3 gm. of the enzyme per 100 gm. of dried venom, and therefore serve as convenient sources of the enzyme. Singer and Kearney isolated the moccasin venom L-amino acid oxidase by ammonium sulfate fractionation and by adsorption on and elution from calcium phosphate gel; the final purification step involved an electrophoretic isolation of the enzyme in about a 50 percent yield. The enzyme was homogeneous in the ultracentrifuge; and was reported to have a molecular weight of 62,000 and 1 mole of FAD per mole of protein. The specific activity toward L-leucine was 6,300.[6]

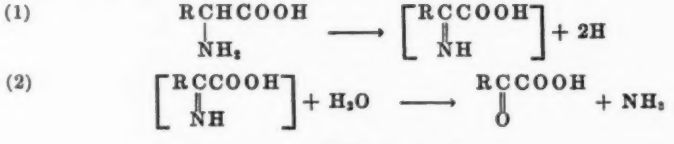
Recent studies in our laboratory (*33, 34*) have led to the isolation of the L-amino acid oxidase of rattlesnake, *Crotalus adamanteus*, venom in crystalline form. The preparation we originally obtained was shown to be homogeneous in the ultracentrifuge, but exhibited three components on

[6] Specific activity is expressed in terms of μl. of oxygen consumed per ml. of enzyme in 30 minutes at 38° C. in a reaction mixture (3.0 ml.) containing enzyme, L-leucine (21 μmoles), and 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl buffer (pH 7.2; 200 μmoles) divided by the absorbancy of the enzyme solution at 275 or 280 mμ in a 1-cm. cell.

electrophoresis. However, each of these electrophoretically separable fractions was found to possess L-amino acid oxidase activity and to exhibit a specific activity of about the same value as that of the original preparation (specific activity, 6,000 to 7,000). The three L-amino acid oxidases were separated also by column electrophoresis, and one of these components was obtained in pure form from the mixture by crystallization under special conditions (*35*). The rattlesnake venom L-amino acid oxidases possess a molecular weight of approximately 133,000 and contain 2 moles of FAD per mole of protein. It is particularly interesting, however, that 3 electrophoretically separable forms of the enzyme occur in rattlesnake venom. In a recent study of venom collected from a single snake, only 2 of these components were found. Furthermore the relative amounts of these components appear to vary with different batches of pooled rattlesnake-venom starting material. This variability may be related to genetic differences; however, other explanations are not excluded. Several L-amino acid oxidases may also be present in moccasin venom. In any event, the multiplicity of L-amino acid oxidases derived from the same source represents a phenomenon that must be taken into account in studies on the structure of these molecules. Structural studies on one of the purified L-amino acid oxidases of rattlesnake venom are in progress.

Investigations of the Mechanism of Amino Acid Oxidation

The view that the oxidation of amino acids by the amino acid oxidases involves formation of an imino acid intermediate, as described in text-figure 1, has not yet been substantiated by isolation of the proposed intermediate. Although there is much evidence consistent with this proposal, the available data appear to be stronger in excluding mechanisms that require an α,β-unsaturated intermediate than in proving that an imino acid intermediate is involved.



TEXT-FIGURE 1

The observations that the isomers of α-aminophenylacetic acid are oxidized by the respective amino acid oxidases (*4*, *6*) would seem to exclude, in the oxidation of this amino acid, an intermediate involving α,β-unsaturation. Studies which demonstrate the conversion of amino acids, with α- and β-centers of asymmetry, to pure optically active products also support the view that amino acid oxidation does not involve α,β-unsaturation. Thus, the four isomers of isoleucine are converted by the respective amino acid oxidases to the corresponding α-keto-β-methylvaleric acids (*36*); this forms the basis of a method for the preparation of the optical isomers of this keto acid (*37*), and Greenstein used this reaction

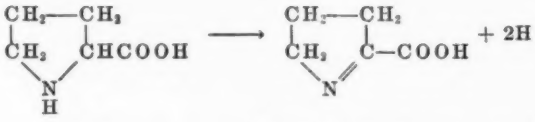
for the characterization of the isomers of isoleucine obtained by enzymatic resolution (*38*). Fones (*39*) carried out the enzymatic oxidation of the L-isomers of β-phenylserine to the corresponding L- and D-mandelic acids (in the absence of catalase), and again demonstrated that no α,β-unsaturation occurred during the enzymatic reaction or the subsequent nonenzymatic oxidative decarboxylation. More recent studies of Frieden and Velick (*40*) have shown that the oxidation of L-leucine by L-amino acid oxidase in D_2O gives α-ketoisocaproic acid containing much less than one mole of deuterium. These results are also inconsistent with a mechanism requiring α,β-unsaturation, but again do not prove the existence of an imino acid intermediate. Pitt (*41*) has recently observed a transient increase in absorption at 300 mμ during the course of the oxidation of L-tyrosine by crude moccasin venom in the presence of an enzyme previously reported to catalyze enolization of *p*-hydroxyphenylpyruvic acid; the absorption observed is considered to represent an enamine derivative formed by action of the tautomerase on the imino acid intermediate. Such an interpretation rests on the assumption that the tautomerase would be capable of acting on the hypothetical intermediate. Proof of this assumption might be no less difficult than isolation and identification of the presumed intermediate itself.

In earlier work by Taborsky (*42*), spectrophotometric observations made during the oxidation of tyrosine by L-amino acid oxidase also showed increase in absorption at 300 mμ, though no tautomerase was added in these experiments. These results were interpreted to indicate the existence of an α,β-unsaturated intermediate. It is, of course, possible that certain amino and keto acids undergo α,β-unsaturation during or after the reaction. Apparently, enolization of the keto acid formed by amino acid oxidation can occur in certain cases. Although this was excluded in the case of the oxidation of the L- and D-isoleucine isomers, where enolization is equivalent to racemization, Frieden and Velick (*40*) found evidence for some enolization of α-ketoisocaproate formed by oxidation of L-leucine. The formation of a new absorption band during the enzymatic hydrolysis (in borate, but not in phosphate buffer) of glycyldehydrophenylalanine has been interpreted as evidence for the occurrence of an imino acid intermediate (*43*). We have observed a similar intermediate absorption curve during the oxidation of L-phenylalanine and L-tyrosine by L-amino acid oxidase in the presence of borate buffer, but not with phosphate buffer of the same value of *p*H. It appears probable that the new absorption peak is due to the complex formed by reaction of borate and the enolic form of the α-keto acid. This phenomenon has been extensively studied by Knox and Pitt (*44*) and used by them and others for the determination of transaminase and other enzymatic activities involving aromatic amino acids (*45*).

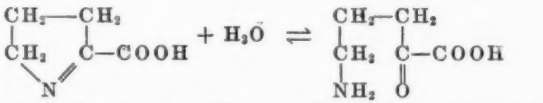
Studies in our laboratory on the oxidation of proline appear to offer a more direct approach to this problem, since the proposed imino acid intermediate in the oxidation of proline, Δ^1-pyrroline-2-carboxylic acid has actually been prepared (*46*), and may therefore be employed in enzymatic

studies. According to the general mechanism (text-fig. 1) postulated for the amino acid oxidase reaction, oxidation of proline would take place as shown in text-figure 2. The imino acid formed by enzymatic dehydrogenation would be expected to hydrolyze to α-keto-δ-aminovaleric acid. Δ^1-Pyrroline-2-carboxylic acid was prepared by enzymatic oxidation of δ-N-carbobenzoxy-α,δ-diaminovaleric acid to the corresponding α-keto acid, followed by removal of the carbobenzoxy group with hydrogen bromide in glacial acetic acid. The compound in solution appeared to be in equilibrium with the corresponding open-chain form (*46*).

The availability of synthetic Δ^1-pyrroline-2-carboxylic acid, the presumed intermediate in the oxidation of proline, made possible a direct examination of its participation in the amino acid oxidase reaction (*47*). Thus if Δ^1-pyrroline-2-carboxylic acid were actually an intermediate in proline oxidation, and if reaction (3) (text-fig. 2) were reversible, it should be possible to demonstrate the synthesis of D-proline from Δ^1-pyrroline-2-carboxylic acid. Reversibility was demonstrated by incubating anaerobically Δ^1-pyrroline-2-carboxylic acid with D-amino acid oxidase

(3) 

Proline Δ^1-Pyrroline-2-carboxylic acid

(4) 

Δ^1-Pyrroline-2-carboxylic acid α-Keto-δ-aminovaleric acid

TEXT-FIGURE 2

and any one of a number of D-amino acid substrates. The experimental findings (*47*, *48*) may be interpreted in terms of the following reactions in which the enzyme, reduced by D-amino acid substrate, reacts with the added imino acid to yield D-proline:

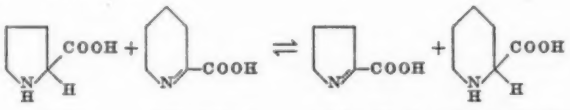
(5) D-oxidase-FAD + D-amino acid + $H_2O \rightleftharpoons$ α-keto acid + NH_3 + D-oxidase-$FADH_2$

(6) D-oxidase-$FADH_2$ + Δ^1-pyrroline-2-carboxylate $\rightleftharpoons$ D-proline + D-oxidase-FAD

That the synthesis of proline was enzymatic was shown by use of appropriate controls without enzyme and with heat-inactivated enzyme, and also by the fact that the proline formed was of the D-configuration. Only D-amino acids that were susceptible to D-amino acid oxidase in the usual oxidative reaction served as hydrogen donors in the D-proline-synthesizing system; thus D-glutamic acid and a number of L-amino acids were inactive. It is of interest that D-allohydroxyproline and D-pipecolic acid served as

hydrogen donors in this system, so that the following over-all reactions could be demonstrated:

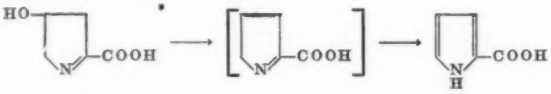
(7)



(8)

Reaction (7), which exhibits an equilibrium constant of approximately 6 at *p*H 6.8 and 37° C., clearly demonstrated that the enzyme catalyzes reversible dehydrogenation. Studies on Δ¹-pyrroline-5-carboxylic acid and the homologous piperidine derivative [α-aminoadipic-δ-semialdehyde; Δ¹-piperidine-6-carboxylic acid (*49*)] have indicated that these isomeric forms are not active in reaction (7). Reaction (8) gives Δ¹-pyrroline-4-hydroxy-2-carboxylic acid, an intermediate also observed in the oxidative reaction with the D-isomers of hydroxyproline (*50*). This intermediate was shown earlier in this laboratory (*50*) to be spontaneously converted to pyrrole-2-carboxylic acid:

(9)



It has been shown by Adams (*51*) that Δ¹-pyrroline-4-hydroxy-2-carboxylic acid is also an intermediate in the oxidation of D-allohydroxyproline by an enzyme of *Pseudomonas*. It was suggested earlier (*50*) that this compound might undergo enzymatic transformation in addition to nonenzymatic conversion to pyrrole-2-carboxylic acid. Adams has presented evidence suggesting that Δ¹-pyrroline-4-hydroxy-2-carboxylic acid is an intermediate in the conversion of hydroxyproline to glutamic acid in hydroxyproline-adapted *Pseudomonas*. Of much interest is the finding by Adams (*51*) that the first step in the metabolism of L-4-hydroxyproline by his strain of *Pseudomonas* is enzymatic epimerization of this amino acid to D-allohydroxyproline.

The formation of pyrrole-2-carboxylic acid, which has been demonstrated also in studies on intact rats (*52*), appears to be a nonenzymatic product of Δ¹-pyrroline-4-hydroxy-2-carboxylic acid. Pyrrole-2-carboxylic acid may be detected in acid solution by the highly sensitive color reaction with *p*-dimethylaminobenzaldehyde. This reaction may be used to determine D-amino acid oxidase, with D-allohydroxyproline as the substrate. A simple and sensitive method for the determination of D-amino acid oxidase is therefore available; the procedure is about 50 times more sensitive than the usual Warburg manometric technique. Some data obtained

TABLE 1.—Comparison of methods for amino acid oxidase determination

Method	Quantity of enzyme (*ml.*)*	Substrate: Allohydroxyproline	Substrate: Phenylalanine
Oxygen uptake†	0.50	72 μl.O_2	134 μl.O_2
Ehrlich's reagent‡	0.01	Absorbancy = 0.132	—
Borate-enol§	0.01	—	Absorbancy = 0.279

*One gm. of hog-kidney acetone powder was extracted for 30 min. with 20 ml. of 0.02 M sodium pyrophosphate buffer (*p*H 8.3), and then centrifuged before use.

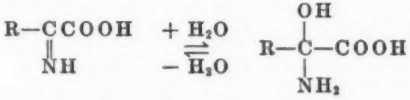
†The Warburg vessels contained 100 μmoles of sodium pyrophosphate buffer (*p*H 8.3), crystalline bovine liver catalase (50 units), and enzyme in the main compartment; 50 μmoles of D-amino acid in 0.5 ml. was added from the side-arm; final volume, 2.5 ml.; 38° C.; gaseous phase, air; incubation time, 30 min.

‡The reaction mixture, in a final volume of 0.5 ml. (concentrations of all components except enzyme as in footnote †) was shaken for 30 min. at 38° C. in small test tubes. Trichloroacetic acid (50%, 0.1 ml.) was added, and then the mixture was centrifuged. An aliquot of 0.3 ml. was treated with 3.7 ml. of 1 N H_2SO_4 and heated for 5 min. at 70° C. After cooling to 25° C., 1 ml. of 5% *p*-dimethylaminobenzaldehyde in *n*-propanol was added, and after 10 min. the solution was read in a Weston colorimeter with 550-mμ filter; the reading is given in the table.

§An aliquot of 0.3 ml. of the centrifuged reaction mixture (incubated and acidified as in footnote ‡) was treated with 1.7 ml. of a solution containing 1 M borate and 2 M arsenate (*p*H 6.5). After standing for 30 min. at 25° C., the solution was read in a Beckman Model DU spectrophotometer at 300 mμ. The reading is given in the table.

with this method are given in table 1. The procedure offers some advantage over the D-phenylalanine-borate procedure (*44*, *45*), in that there is less possibility of interference by transaminases or by substances that exhibit high ultraviolet absorption in borate.

The studies described, which demonstrate the enzymatic utilization of Δ^1-pyrroline-2-carboxylic acid and Δ^1-piperidine-2-carboxylic acid by amino acid oxidase appear to provide the most direct information now available for the participation of an imino acid intermediate in amino acid oxidation. It is possible that the hydrated form of the imino acid is involved, as has been considered by Hellerman *et al.* (*53*) for the glutamic dehydrogenase system:



It is of further interest that the amino acid oxidases, *both* the D-amino acid oxidase of hog or sheep kidney and the L-amino acid oxidase of moccasin and rattlesnake venoms, catalyze amino acid synthesis from the corresponding α-keto acids and ammonia in the presence of enzyme reduced by adding to the system an amino acid substrate of proper configuration. The results of experiments of this type may be represented by the following equations:

(10) L-Phenylalanine + enzyme-FAD + $H_2O \rightleftharpoons$ enzyme-$FADH_2$ + phenylpyruvate + NH_3

(11) α-Ketoisocaproate + enzyme-$FADH_2$ + $NH_3 \rightleftharpoons$ enzyme-FAD + L-leucine + H_2O

Sum: (12) L-Phenylalanine + α-ketoisocaproate $\rightleftharpoons$ L-leucine + phenylpyruvate

Reaction (12) is formally identical with a transamination reaction. However, that amino group transfer (transamination) is not involved, follows from several lines of evidence (*47*, *48*): (*a*) The over-all reaction requires ammonium ions and is accelerated by increasing the concentration of ammonium ions. (*b*) Incorporation of N^{15}-ammonia into the amino acid has been demonstrated with both L- and D-amino acid oxidases. (*c*) Reactions (10) and (11) have been demonstrated separately by spectrophotometric procedures. Thus, anaerobic reoxidation of reduced D- and L-amino acid oxidases has been observed in the presence of α-keto acid and ammonia (*48*).

Demonstration of these reactions catalyzed by the L- and D-amino acid oxidases, which lead to amino acid synthesis, and the data cited previously, which exclude α, β-unsaturation, give strong support to the concept of an imino acid intermediate. The optical specificity of amino acid synthesis indicates that the intermediate must be bound to the enzyme at some stage. Such an intermediate could be formed in at least three ways:

(*a*) Enzyme + NH_3 → enzyme-NH_3 $\xrightarrow{\text{keto acid}}$ enzyme-imino acid

(*b*) Enzyme + keto acid → enzyme-keto acid $\xrightarrow{NH_3}$ enzyme-imino acid

(*c*) Enzyme + imino acid → enzyme-imino acid

In studies on the formation of L-leucine from L-phenylalanine and α-ketoisocaproate, the concentration of α-ketoisocaproate required for half-maximal rate of leucine synthesis was essentially unaffected by wide variations in ammonia concentration (*48*). These results are consistent with mechanism (*b*); however, present data do not permit an unequivocal choice between these mechanisms of enzyme-imino acid intermediate formation. Further studies are also needed to determine whether ammonium ion or free un-ionized ammonia is the enzymatically active reactant.

The introduction of molecular oxygen into the system requires additional considerations. According to the generally accepted mechanism, molecular oxygen reacts with reduced enzyme to yield hydrogen peroxide and oxidized enzyme, and there is presumably no direct reaction of oxygen with the enzyme-imino acid complex. Oxidation of L-leucine by crystalline L-amino acid oxidase of rattlesnake venom takes place approximately 3 times more rapidly in pure oxygen than in air. Furthermore, marked substrate inhibition of oxidation was observed even at relatively low concentrations of leucine, *e.g.*, 0.0075 M. However, it was observed that the rate of oxidation of L-valine by L-amino acid oxidase was the same in air as in 100 percent oxygen, with concentrations of L-valine of 0.02 M or less. Inhibition by substrate did not occur even with concentrations of L-valine as high as 0.3 M. Experiments on oxidation of L-leucine and L-valine in air and in oxygen are described in table 2. The effects of oxygen tension were independent of enzyme concentration. Analogous results were obtained on the oxidation of D-pipecolic acid and D-proline

TABLE 2. Influence of oxygen tension on oxidation of L-amino acids*

Expt. No.	L-Leucine		L-Valine	
	Air	O_2	Air	O_2
1	235	632	13	12
2	34	96	—	—
3	—	—	194	193

*The reaction mixtures consisted of rattlesnake venom L-amino acid oxidase (20, 2, and 400 μg. in expts. 1, 2, and 3, respectively), L-leucine and L-valine (50 μmoles), 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl buffer (pH 7.8; 180 μmoles) in a final volume of 2.5 ml. Values expressed as microliters of oxygen consumed in 30 min. at 38° C.

by D-amino acid oxidase (table 3). These findings could be interpreted in terms of the classical mechanism:

(13) $$\text{Enzyme-FAD} + \underset{\underset{\text{NH}_2}{|}}{\text{RCHCOOH}} \rightleftharpoons \text{enzyme-FADH}_2 + \left[\underset{\underset{\text{NH}}{\|}}{\text{RCCOOH}}\right]$$

(14) $$\text{Enzyme-FADH}_2 + \text{O}_2 \rightleftharpoons \text{H}_2\text{O}_2 + \text{enzyme-FAD}$$

Thus it might be concluded that reaction (14) is rate-limiting (under the conditions used) with leucine, while reaction (13) is rate-limiting with L-valine. However, this interpretation, although consistent with the observations on the effect of oxygen tension, does not clarify the results of the experiments on the effects of substrate concentration. Inhibition by high concentrations of substrate has been explained previously by Zeller (*26*) in terms of reaction of the enzyme with an additional molecule of substrate to yield an inactive complex. Inhibition of methylene blue reduction by L-amino acid oxidase by high concentrations of L-leucine has been discussed by Dixon and Webb (*54*), who explain the inhibition in terms of competition between leucine and the dye.

An alternative explanation is suggested by our findings on the oxidation of valine and leucine described; this explanation is in accord also with recent experiments by Beinert (*55*) and Commoner *et al.* (*56*), which demonstrate free radical formation in the course of flavoprotein-catalyzed reactions. Beinert (*55*) has found spectrophotometric evidence for transient formation of an intermediate during reduction (by substrate or

TABLE 3.—Influence of oxygen tension on oxidation of D-amino acids*

Expt. No.	DL-Proline		DL-Pipecolic acid	
	Air	O_2	Air	O_2
1	112	273	30	29
2	16	27	—	—
3	—	—	121	124

*The reaction mixtures consisted of purified hog-kidney D-amino acid oxidase (100, 20, and 500 μg. in expts. 1, 2, and 3, respectively), FAD (91% pure; 15 μg.), DL-pipecolic acid and DL-proline (100 μmoles), and sodium pyrophosphate buffer (pH 8.3; 100 μmoles) in a final volume of 2.5 ml.; values expressed as microliters of oxygen consumed in 30 min. at 38° C.

dithionite) of L-amino acid oxidase. The same intermediate appeared during aerobic reoxidation, and it was concluded that the intermediate was a semiquinone form of the flavine.

We may tentatively interpret our experimental data as follows: The initial enzymatic reaction leads to formation of a semiquinone intermediate, which may react either with oxygen to yield oxidized flavoprotein, or with another molecule of substrate to give the completely reduced enzyme. With low substrate concentrations, oxidation of the intermediate would be more rapid than its reduction by substrate. In large concentrations of substrate, a greater proportion of the enzyme will be completely reduced. Since oxygen would be expected to react less readily with completely reduced enzyme than with the free radical, the over-all rate would decrease, as was observed in studies with high concentrations of L-leucine. Increasing the oxygen tension would be expected to reduce such inhibition by increasing the rate of oxidation of the intermediate. With valine, the rate of dehydrogenation is so slow that significant concentrations of the reduced enzyme forms do not accumulate in air. Thus there is less tendency for substrate inhibition to occur and no demonstrable effect of oxygen tension except at relatively high concentrations of substrate. This explanation is summarized by the following tentative scheme:

$$\text{Enzyme-FAD} \xrightarrow{\text{Amino Acid}} \text{[Enzyme-FADH]}$$

$$\text{[Enzyme-FADH]} \xrightarrow{\text{Amino Acid}} \text{Enzyme-FADH}_2$$

$$\text{[Enzyme-FADH]} \xrightarrow{\text{Rapid}} \text{Enzyme-FAD}$$

$$\text{Enzyme-FADH}_2 \xrightarrow[O_2]{\text{Slow}} \text{Enzyme-FAD}$$

This explanation is admittedly incomplete; other interpretations are not yet excluded and additional work is required before a definite conclusion can be reached.

It is evident that a complete understanding of the mechanism of the amino acid oxidase reactions requires an explanation of the strict antipodal specificity of these enzymes. Perhaps information relating to the binding of FAD to the peptide chains of these molecules will provide clues as to the geometry of the enzyme-prosthetic group structure associated with such specificity.

As Greenstein and his collaborators have shown (*57*), the amino acid oxidases also exhibit a relative specificity for the configuration of the β-carbon atom. Thus L-amino acid oxidase oxidizes L-isoleucine (whose β-carbon atom is of the L-configuration) more rapidly than L-alloisoleucine, but L-allothreonine is a better substrate than is L-threonine (whose β-carbon atom is of the D-configuration). Analogous findings were made with the D-amino acid oxidase (*57*).

Additional problems relating to the specificity of these enzymes also remain to be solved. For example, the sheep-kidney D-amino acid

oxidase and the rattlesnake L-amino acid oxidase do not exhibit entirely similar substrate specificity; thus the kidney D-amino acid oxidase attacks D-proline and *N*-methyl-D-amino acids, while the snake venom L-amino acid oxidase does not attack the corresponding L-isomers but does oxidize L-glutamine and L-asparagine, whose D-enantiomorphs are not substrates for D-amino acid oxidase. Neither enzyme attacks lysine or glutamic acid at significant rates, although other oxidases, as noted previously, oxidize these amino acids.

Physiological Significance of the Amino Acid Oxidases

The prominent role of transamination in amino acid metabolism has been emphasized and is widely accepted (*58*). It seems probable, however, that oxidative deamination has a function in the deamination of certain amino acids, that different pathways of deamination may exist in different cells, and that some cells may have more than one mechanism for the conversion of a given amino acid to the corresponding α-keto acid. Although deamination by coupled reactions catalyzed by glutamic dehydrogenase and glutamic transaminase (*59*) may represent a significant pathway for deamination of amino acids in mammalian tissues, the available evidence does not exclude the occurrence of oxidative deamination of certain amino acids. Transamination of lysine has not been demonstrated in mammalian tissues, and it is possible that this amino acid is oxidatively deaminated, perhaps by an enzyme similar to the basic amino acid oxidase described by Boulanger and Osteux (*27–29*). The function of Green's L-amino acid oxidase (*20, 21*) is still not clear and the possibility exists that the isolated L-amino acid oxidase is a fragment of a more complex system coupled to hydrogen or oxygen transport systems. It would be desirable tc reinvestigate this enzyme.

Oxidative deamination of L-amino acids may be more significant in certain lower forms, while transamination is more prominent in mammals in which apparently the concentration of free ammonia is maintained at a relatively low value. Some microorganisms such as *P. vulgaris* (*24*) catalyze relatively rapid amino acid oxidation, while in *Escherichia coli* and certain other microorganisms transamination appears to be more prominent than oxidative deamination. The function of L-amino acid oxidase in the venom and tissues of snakes is not known; Zeller has discussed this problem in detail (*26*).

Although the function of D-amino acid oxidase in microorganisms may be related to the presence of D-amino acids in the cells, we know relatively little about the metabolism of the D-isomers of amino acids. A number of other enzymes that act on D-amino acids, *e.g.*, transaminase, racemase, asparaginase, cysteine desulfhydrase, transpeptidase, have been discovered recently. One can only speculate as to the possible function of D-amino acid oxidase in the synthesis of D-amino acids in microorganisms. The synthesis of amino acids observed in isolated systems requires a source of reduced enzyme, anaerobiosis, and a fairly high concentration of ammonium ions. Whether appropriate conditions for synthesis can be achieved

under physiological conditions is not certain, though it is conceivable that the localization of the enzyme within the cell structure might in some way provide conditions favorable for synthesis.

The physiological function of mammalian D-amino acid oxidase (or the explanation for its presence in the mammal) has remained obscure since this enzyme was discovered in mammalian liver and kidney 27 years ago. Although the discovery of an enzyme without a physiological function is not unique, the "unnatural" characteristics of its substrates, and its widespread occurrence in the tissues of mammals and some other animals, serve to make the reason for its presence in mammals an intriguing problem. There are at least four categories of explanations for the presence of this enzyme:

1. D-Amino acid oxidase may be an artifact of isolation. In the course of isolation, this enzyme might be formed by alteration of another protein or of an enzyme that may possess a different substrate specificity *in vivo*.

2. The enzyme may have a natural substrate not yet discovered. The enzyme's action on D-amino acids may thus be explained by some relationship or similarity between the structure of D-amino acids and the natural, as yet undiscovered, substrate.

3. The enzyme has no physiological function but exists in mammals as a vestigial enzyme.

4. The physiological function of D-amino acid oxidase is to destroy D-amino acids which are (*a*) ingested by the mammal, (*b*) formed by the mammal's bacterial flora, or (*c*) formed in mammalian tissues by other reactions.

It appears unlikely that D-amino acid oxidase is an artifact of isolation. The utilization of the D-isomers of the essential amino acids by mammals is consistent with physiologically active D-amino acid oxidase. That certain amino acids, *e.g.*, D-histidine and D-tryptophan, are less readily used by the mouse (*60*) than the rat (*61–63*) correlates well with the negligible D-amino acid oxidase of mouse liver (*64*). Nephrectomized mice, in contrast to a sham-operated mouse, utilized administered C^{14}-D-alanine only slightly (*65*). Little can be said concerning the second or third possibilities mentioned, except perhaps to note that if the "vestigial" explanation is correct, we must still seek the function performed by this enzyme in an earlier evolutionary stage. The possibility that the "true" substrate of D-amino acid oxidase is not yet known cannot be discarded; there have been other instances in which an enzyme was discovered prior to the discovery of its natural substrate.[7]

Perhaps the fourth possibility is most plausible. The discovery of D-amino acids in microorganisms and in other lower forms makes it reasonable to assume that D-amino acids may occasionally find their way into the

[7]An interesting example is "acylpyruvase," which was discovered in 1947 in Greenstein's laboratory. The enzyme was first recognized by its catalysis of the hydrolysis of acylpyruvic acids, *e.g.*, acetopyruvic acid, to pyruvic acid and the corresponding fatty acids (*66*). Subsequently it was shown that the enzyme hydrolyzed triacetic acid (3,5-dioxohexanoic acid) to acetoacetic acid and acetic acid (*67, 68*). Later (*69*) it was found that the enzyme hydrolyzed fumarylacetoacetic acid (now believed to be the natural substrate) to fumaric acid and acetoacetic acids.

intestinal tracts of mammals. Thus mammals may ingest materials containing D-amino acids, and D-amino acids produced by the bacterial flora may enter mammalian blood and tissues. D-Amino acids might occasionally be formed in mammalian tissues by nonenzymatic transamination or racemization, or perhaps by reactions not yet discovered. Reactions of the type observed during hydrolysis of Bacitracin A (*70*) might occur, in which formation of a thiazoline ring apparently leads to epimerization of an N-terminal isoleucine residue, thus yielding D-alloisoleucine.

The production of D-amino acids by the bacterial flora could be quantitatively significant, and if the formation of D-amino acids were adaptive in nature, one might expect the D-amino acid oxidase activity of the liver and kidney to increase rapidly after birth in response to development of the bacterial flora. Studies in this laboratory have revealed that, although fetal rat liver and kidney exhibit D-amino acid oxidase activity, the activity per gram of tissue is only about 10 percent of that of the livers and kidneys of rats, 1 month of age. The D-amino acid oxidase activity of both rat kidney and liver increases rapidly after birth, reaching a peak when the animals are about 25 to 35 days of age; the tissues of animals 45 to 61 days old exhibited somewhat less activity. Injection or oral administration of D-amino acids to very young rats did not significantly affect the development of D-amino acid oxidase activity.

TABLE 4.—D-Amino acid oxidase activity of the kidneys of germ-free mice*

Mouse No.	Sex	Age (*days*)	Kidney weight (*gm.*)	D-Amino acid oxidase
		Germ-free animals		
1	♀	190	0. 588	10. 9
2	♀	190	. 551	9. 39
3	♂	40	. 424	31. 9
4	♀	40	. 325	22. 2
5	♀	190	. 215	10. 4
6	♂	40	. 221	27. 9
7	♀	40	. 128	18. 3
8	♂	15	. 052	11. 6
9	♂	15	. 066	10. 0
10	♂	15	. 064	10. 1
11	♀	15	. 050	7. 55
		Nonsterile animals		
1	♀	193	0. 262	14. 9
2	♀	162	. 398	15. 3
3	♀	128	. 162	12. 6
4	♂	101	. 113	15. 6
5	♀	70	. 257	12. 6
6	♂	41	. 184	13. 1
7	♀	11	. 089	11. 9

*The reaction mixtures used for enzyme assay consisted of 0.1 ml. of kidney homogenate (1 part tissue plus 24 parts of 0.1 M sodium pyrophosphate buffer (*p*H 8.3), D-phenylalanine (10 μmoles), 5 units of crystalline bovine liver catalase, 5 μg. of FAD, and 50 μmoles of sodium pyrophosphate buffer (*p*H 8.3) in a final volume of 0.74 ml.; shaken at 37° C. in air for 15 minutes. Values expressed as μmoles of phenylpyruvate formed per hour per ml. of homogenate.

A test of the hypothesis that D-amino acid oxidase might be formed adaptively in response to D-amino acids produced by the bacterial flora was carried out by examining the kidneys of a number of germ-free mice, supplied by Dr. Walter Newton of the National Institutes of Health. As indicated in table 4, the kidneys of the germ-free mice exhibited appreciable D-amino acid oxidase activity; the activity was comparable to that of the kidneys of mice raised under nonsterile conditions. In confirmation of Shack's findings (*64*), negligible amounts of D-amino acid oxidase activity were found in the livers of both groups of mice.

Although these results appear to show that D-amino acid oxidase does not develop adaptively to bacterial D-amino acids, they do not necessarily exclude the possibility that the enzyme has a physiological function related in some way to the presence of D-amino acids in nature. Perhaps more information concerning D-amino acids is needed before this problem can be solved. Whether or not this enzyme contributes to the complex biochemical phenomena associated with cellular activity, it cannot be denied that it has performed a number of important practical tasks for the biochemist.

REFERENCES

(*1*) WARBURG, O., and CHRISTIAN, W.: Co-Ferment der *d*-Alanin-Oxydase. Biochem. Ztschr. 296: 294, 1938.

(*2*) ———: Isolierung der prosthetischen Gruppe der *d*-Aminosäureoxydase. Biochem. Ztschr. 298: 150–168, 1938.

(*3*) GREENSTEIN, J. P.: The resolution of racemic α-amino acids. Advances Protein Chem. 9: 121–202, 1954.

(*4*) MEISTER, A., LEVINTOW, L., KINGSLEY, R. B., and GREENSTEIN, J. P.: Optical purity of amino acid enantiomorphs. J. Biol. Chem. 192: 535–541, 1951.

(*5*) BRAUNSTEIN, A. E., and BYCHKOV, S. N.: Cell-free enzymic model of L-amino acid dehydrogenase (L-deaminase). Nature, London 144: 751, 1939.

(*6*) KREBS, H. A.: Metabolism of amino acids. III. Deamination of amino acids. Biochem. J. 29: 1620–1644, 1935.

(*7*) DAS, N. B.: Studies on amino-acid dehydrogenase. I. Proline dehydrogenase. Biochem. J. 30: 1080–1087, 1936.

(*8*) BERNHEIM, F., BERNHEIM, M. L. C., and WEBSTER, M. D.: Oxidation of certain amino acids by "resting" Bacillus proteus. J. Biol. Chem. 109: VI, 1935.

(*9*) NEGELEIN, E., and BRÖMEL, H.: Protein der *d*-Aminosäureoxydase. Biochem. Ztschr. 300: 255–239, 1939.

(*10*) VEITCH, F. P., and MCCOMB, R.: The purification of hog kidney D-amino acid oxidase. J. Am. Chem. Soc. 78: 1363–1367, 1956.

(*11*) KUBO, H., YAMANO, T., IWATSUBO, M., WATARI, H., SOYAMA, T., SHIRAISHI, J., SAWADA, S., KAWASHIMA, N., MITANI, S., and ITO, K.: Sur l'interaction entre l'enzyme et le co-enzyme dans le système de la déshydrogénase de l'acide glutamique et celui de l'oxydase des acides aminés. (Interaction of enzyme and coenzyme of the glutamic acid dehydrogenase system and oxidases of amino acids.) Bull. Soc. chim. Biol. 40: 431–447, 1958.

(*12*) BLASCHKO, H., and HAWKINS, J.: D-Amino-acid oxidase in the molluscan liver. Biochem. J. 52: 306–310, 1952.

(*13*) KREBS, H. A.: The Relation of Optical Form to Biological Activity in the Amino-Acid Series. Biochem. Soc. Symposia. Cambridge, England, No. 1, 1948.

(14) HOROWITZ, N. H.: The D-amino acid oxidase of neurospora. J. Biol. Chem. 154: 141–149, 1944.
(15) EMERSON, R. L., PUZISS, M., and KNIGHT, S. G.: The D-amino acid oxidase of molds. Arch. Biochem. 25: 299–308, 1950.
(16) MIZUSHIMA, S., and SAKAGUCHI, K.: Metabolism of D-amino acids in microorganisms. III. Presence of D-monoaminodicarboxylic acid oxidase. Bull. Agr. Chem. Soc. (Japan) 20: 131, 1956.
(17) STILL, J. L., BUELL, M. V., KNOX, W. E., and GREEN, D. E.: Studies on the cyclophorase system. VII. D-Aspartic oxidase. J. Biol. Chem. 179: 831–837, 1949.
(18) STILL, J. L., and SPERLING, E.: On the prosthetic group of the D-aspartic oxidase. J. Biol. Chem. 182: 585–589, 1950.
(19) ROCCA, E., and GHIRETTI, F.: Purification and properties of D-glutamic acid oxidase from *Octopus vulgaris* Lam. Arch. Biochem. 77: 336, 1958.
(20) BLANCHARD, M., GREEN, D. E., NOCITO, V., and RATNER, S.: L-Amino acid oxidase of animal tissue. J. Biol. Chem. 155: 421–440, 1944.
(21) ———: Isolation of L-amino acid oxidase. J. Biol. Chem. 161: 583–597, 1945.
(22) THAYER, P. S., and HOROWITZ, N. H.: The L-amino acid oxidase of neurospora. J. Biol. Chem. 192: 755–767, 1951.
(23) BURTON, K.: The L-amino-acid oxidase of *neurospora*. Biochem. J. 50: 258–268, 1952.
(24) STUMPF, P. K., and GREEN, D. E.: L-Amino acid oxidase of Proteus vulgaris. J. Biol. Chem. 153: 387–399, 1944.
(25) KNIGHT, S. G.: The L-amino acid oxidase of molds. J. Bact. 55: 401–407, 1948.
(26) ZELLER, E. A.: Enzymes of snake venoms and their biological significance. Advances Enzymol. 8: 459–495, 1948.
(27) BOULANGER, P., OSTEUX, R., and BERTRAND, J.: Désamination de l'hydroxylysine par la L-aminoacide-deshydrogénase du foie de dindon (*Meleagris gallopavo* L). Biochim. et biophys. acta 29: 534–536, 1958.
(28) BOULANGER. P., BERTRAND, J., and OSTEUX, R.: Désamination de l'ornithine et de la lysine sélectivement marquées par la L-aminoacide-déshydrogénase du foie de dindon (*Meleagris gallopavo* L). Biochim. et biophys. acta 26: 143–145, 1957.
(29) BOULANGER, P., and OSTEUX, R.: Action de la L-aminoacide-déshydrogénase du foie de dindon (*Meleagris gallapavo* L) sur les acides aminés basiques. Biochim. et biophys. acta 21: 552–561, 1956.
(30) ZELLER, E. A., and MARITZ, A.: Über eine neue L-aminosäure-oxydase. Helv. chim. acta 27: 1888–1902, 1944.
(31) SINGER, T. P., and KEARNEY, E. B.: The L-amino acid oxidases of snake venom. I. The prosthetic group of the L-amino acid oxidase of moccasin venom. Arch. Biochem. 27: 348–363, 1950.
(32) ———: The L-amino acid oxidases of snake venom. II. Isolation and characterization of homogeneous L-amino acid oxidase. Arch. Biochem. 29: 190–209, 1950.
(33) WELLNER, D., and MEISTER, A.: Crystalline L-amino acid oxidase. *In* Abstracts, 4th International Congress of Biochemistry. Vienna, Austria, 1958.
(34) ———: Properties of crystalline L-amino acid oxidase from *Crotalus adamanteus*. *In* Abstracts, Am. Chem. Soc. Div. Biol. Chem., April, 1959, p. 10c.
(35) ———: Unpublished data.
(36) MEISTER, A.: Enzymatic conversion of the stereoisomers of *iso*leucine to the corresponding α-keto acids. Nature, London 168: 1119, 1951.
(37) ———: Studies on *d* and *l*-α-keto-β-methylvaleric acids. J. Biol. Chem. 190: 269–276, 1951.
(38) GREENSTEIN, J. P., LEVINTOW, L., BAKER, C. G., and WHITE, J.: Preparation of the four stereoisomers of isoleucine. J. Biol. Chem. 188: 647–663, 1951.

(39) FONES, W. S.: The enzymatic conversion of L-threo and L-erythro-β-phenylserine to D- and L-mandelic acid. Arch. Biochem. 36: 486–487, 1952.

(40) FRIEDEN, C., and VELICK, S. F.: Imino acid as the intermediate in the L-amino acid oxidase reaction. Biochim. et biophys. acta 23: 439–440, 1957.

(41) PITT, B. M.: The formation of α-imino acids in the enzymatic oxidation of amino acids. J. Am. Chem. Soc. 80: 3799–3800, 1958.

(42) TABORSKY, G.: Mechanism of the oxidation of tyrosine by amino acid oxidase of snake venoms. Yale J. Biol. & Med. 27: 267–278, 1955.

(43) SHACK, J.: Intermediate in the hydrolysis of glycylhydrophenylalanine by dehydropeptidase I. Arch. Biochem. 17: 199–201, 1948.

(44) KNOX, W. E., and PITT, B. M.: Enzymic catalysis of the keto-enol tautomerization of phenylpyruvic acids. J. Biol. Chem. 225: 675–688, 1957.

(45) ASPEN, A. J., and MEISTER, A.: Determination of transaminase. *In* Methods of Biochemical Analysis (Glick, D., ed.). New York, Interscience Publishers, Inc., vol. 6, 1958, p. 132.

(46) MEISTER, A.: The α-keto analogues of arginine, ornithine, and lysine. J. Biol. Chem. 206: 577–585, 1954.

(47) RADHAKRISHNAN, A. N., and MEISTER, A.: Synthesis of amino acids catalyzed by amino acid oxidases. J. Am. Chem. Soc. 79: 5828–5829, 1957.

(48) ———: Amino acid synthesis by reversal of the amino acid oxidase reaction. J. Biol. Chem. 233: 444–450, 1958.

(49) ASPEN, A. J., and MEISTER, A.: α-Aminoadipic acid -δ-semialdehyde. *In* Abstracts, Am. Chem. Soc. Div. Biol. Chem., April, 1959, p. 62c–63c.

(50) RADHAKRISHNAN, A. N., and MEISTER, A.: Conversion of hydroxyproline to pyrrole-2-carboxylic acid. J. Biol. Chem. 226: 559–571, 1957.

(51) ADAMS, E.: Hydroxyproline metabolism. I. Conversion to α-ketoglutarate by extracts of *Pseudomonas*. J. Biol. Chem. 234: 2073–2084, 1959.

(52) WOLF, G., HECK, W. W., and LEAK, J. C.: The metabolism of hydroxyproline-α-C^{14} in the intact rat. Radioactivity in amino acids from proteins. J. Biol. Chem. 223: 95–105, 1956.

(53) HELLERMAN, L., SCHELLENBERG, K. A., and REISS, O. K.: L-Glutamic acid dehydrogenase. J. Biol. Chem. 233: 1468–1478, 1958.

(54) DIXON, M., and WEBB, E. C.: Enzymes. New York, Academic Press, Inc., 1958, p. 87.

(55) BEINERT, H.: Evidence for an intermediate in the oxidation-reduction of flavoproteins. J. Biol. Chem. 225: 465–478, 1957.

(56) COMMONER, B., LIPPINCOTT, B. B., and PASSONEAU, J. V.: Electron-spin resonance studies of free-radical intermediates in oxidation-reduction enzyme systems. Proc. Nat. Acad. Sc. 44: 1099–1110, 1958.

(57) WINITZ, M., BLOCH-FRANKENTHAL, L., IZUMIYA, N., BIRNBAUM, S. M., BAKER, C. G., and GREENSTEIN, J. P.: Studies on diastereoisomeric α-amino acids and corresponding α-hydroxy acids. VII. Influence of β-configuration on enzymatic susceptibility. J. Am. Chem. Soc. 78: 2423–2430, 1956.

(58) MEISTER, A.: Transamination. Advances Enzymol. 16: 185, 1955.

(59) BRAUNSTEIN, A. E.: Les voies principales de l'assimilation et dissimilation de l'azote chez les animaux. Advances Enzymol. 19: 335, 1957.

(60) BAUER, C. D., and BERG, C. P.: The amino acids required for growth in mice and the availability of their optical isomers. J. Nutrition 26: 51–63, 1943.

(61) COX, G. J., and BERG, C. P.: The comparative availability of D- and L-histidine for growth. J. Biol. Chem. 107: 497–503, 1934.

(62) BERG, C. P.: Tryptophane metabolism. IV. The influence of optical activity on the utilization of tryptophan for growth and for kynurenic acid production. J. Biol. Chem. 104: 373–384, 1934.

(63) OESTERLING, M. J., and ROSE, W. C.: Tryptophan requirement for growth and utilization of its optical isomers. J. Biol. Chem. 196: 33–43, 1952.

(64) SHACK, J.: Cytochrome oxidase and *d*-amino acid oxidase in tumor tissue. J. Nat. Cancer Inst. 3: 389–396, 1943.

(65) PASSMANN, J. M., COOPER, J. A. D., and RADIN, W. S.: Studies of D-amino acid oxidase. Cancer Res. 17: 1077–1081, 1957.

(66) MEISTER, A., and GREENSTEIN, J. P.: Enzymatic hydrolysis of 2,4-diketo acids. J. Biol. Chem. 175: 573–588, 1948.

(67) WITTER, R. F., and STOTZ, E.: The metabolism in vitro of triacetic acid and related diketones. J. Biol. Chem. 176: 501–510, 1948.

(68) MEISTER, A.: Metabolism of 3,5-diketohexanoic acid and its δ-lactone by tissue homogenates. J. Biol. Chem. 178: 577–589, 1949.

(69) RAVDIN, R. G., and CRANDALL, D. I.: The enzymatic conversion of homogentisic acid to 4-fumarylacetoacetic acid. J. Biol. Chem. 189: 137–149, 1951.

(70) ABRAHAM, E. P.: Biochemistry of some peptide and steroid antibiotics. *In* Ciba Lectures in Microbial Biochemistry. New York, John Wiley & Sons, Inc., 1957.

Further Development of Manometric Methods [1]

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SUMMARY

The utilization of vessels with a centrally incorporated trough has been applied to the further development of manometry.—J. Nat. Cancer Inst. 24: 51–55, 1960.

AS SEEN in the manometric vessel shown in figure 1, a trough is connected with the side-arm, the cell suspension is placed in the main compartment, and in the trough and side-arm are placed 0.2 cc. of neutral solutions of $KMnO_4$ and $K_4Fe(CN)_6$, respectively, which, when mixed, produce KOH:

$$KMnO_4 + 3[K_4Fe(CN)_6] + 2H_2O = MnO_2 + 3[K_3Fe(CN)_6] + 4KOH.$$

$KMnO_4$ in combination with the reducing agent NaI has been recommended previously by Krebs (*1*) for the same purpose, but was disadvantageous because of the formation of hypoiodide. In comparison with the vessels that I had previously suggested to Dickens (*2–5*) for determining CO_2, the vessel pictured in figure 1 has the advantage that the entire bottom of the main compartment remains available for the cell suspension, and the alkali does not move in a ring on the bottom but—much more actively—in the center of the vessel.

When the solutions in the side-arm and trough are mixed at time t, one obtains the partial pressure of CO_2 in the vessel at time t; and if the partial pressure of CO_2 has been determined in the same way at time t_0, one has the change in partial pressure of CO_2, h_{CO_2}, for the time $(t - t_0)$, and can calculate from this the exchange of CO_2 and O_2 for the time $(t - t_0)$:

$$x_{CO_2} = h_{CO_2} \times k_{CO_2}$$

$$x_{O_2} = (H - h_{CO_2}) \times k_{O_2}$$

$$H = h_{CO_2} + h_{O_2} \text{ in the time } (t - t_0),$$

where k_{CO_2} and k_{O_2} are the vessel constants for CO_2 and O_2.

[1] Received for publication August 3, 1959.

The amounts of reagents put into the trough and side-arm are generally 2.6 mg. $KMnO_4$ in 0.2 cc. H_2O = 16.7 μmoles in the trough, and 21 mg. $K_4Fe(CN)_6$ in 0.2 H_2O = 50 μmoles in the side-arm. These amounts are sufficient to absorb more than 1000 mm.^{3} CO_2. When the amounts of CO_2 are considerably larger, the amounts of the reagents are increased accordingly.

As fluid for the manometer capillary arms, we use Brodie's solution up to CO_2 pressures of 200-mm. Brodie, and for higher CO_2 pressures mercury has proved effective with capillary diameters from 0.8 to 1 mm. Naturally, the exchange of CO_2 and O_2 must always be so selected by variation of the amounts of cells and the experimental times that the deflections in millimeters Brodie or millimeters mercury are large enough.

But if the partial pressures of CO_2 in the vessel are too large and the partial pressures of O_2 are small, it is often advisable to determine the O_2 instead of the CO_2, by placing 0.4 cc. water in the trough and 20 mg. dry $Na_2S_2O_4$ and 20 mg. dry $CaCl_2$ in the side-arm. If the salts are then placed in the trough, the O_2 absorption begins immediately:

$$O_2 + Na_2S_2O_4 = Na_2SO_4 + SO_2,$$

and one obtains h_{O_2} directly, and $h_{CO_2} = H - h_{O_2}$ indirectly.

If the main compartment of the manometer vessel contains respiring cells, the respiration continues after the mixing of contents of the trough and the side-arm. The respiratory CO_2 is absorbed by the KOH in the trough to the same extent that it is given off by the cells in the gas phase, and so does not produce any pressure change. But the consumption of O_2 in respiration produces a negative pressure that must be entered as a correction in the CO_2 determination.

If, for example, there are 50 mm.^{3} of Chlorella in the main compartment of the manometer vessel and a partial CO_2 pressure of 200-mm. Brodie in the gas phase, we find:

Pressure change after mixing the contents of trough and side-arm, final value 10′ after mixing	− 206 mm.
Pressure change in another 5′ as a result of respiration	− 3 mm.
Pressure change in another 5′ as a result of respiration	− 3 mm.
Therefore, partial pressure of CO_2 in the vessel at the time of mixing	+ 200 mm.

DETERMINATION OF BOUND AND FREE CO_2

If the cell suspension contains bicarbonate, the bicarbonate breaks down after mixing the contents of the trough and side-arm because of reduction of the CO_2 pressure according to the equation

$$2\ NaHCO_3 = Na_2CO_3 + H_2O + CO_2,$$

which means that there is now a slow flow of CO_2 from the cell suspension into the trough, with the result that the final manometric value is established only gradually. In such cases we use vessels like that shown in figure 2, which, in addition to the side-arm connected with the trough, contain a second side-arm that is connected with the main compartment. If an excess of sulfuric acid is brought into the main compartment from the second side-arm at time t, one obtains the bicarbonate (bound CO_2) in the vessel at time t; and if the contents of the trough and first side-arm are then mixed, one obtains the total CO_2 present in the vessel, and also the *free* CO_2 present in the vessel from the difference between the *total* and the *bound* CO_2.

REMARKS

1. Vessels like that in figure 1 are especially suitable for determination of *retention* of CO_2, *i.e.*, for determining the CO_2 that is bound as bicarbonate when the CO_2 pressure is increased. For this purpose we place 3 cc. H_2O or 3 cc. of the retaining liquid (*e.g.*, protein solution, phosphate solution) in the main compartment, about 10 μmoles $NaHCO_3$ dissolved in 0.4 cc. H_2O in the trough, about 20 mg. $KHSO_4$ (=about 150 μmoles) in the side-arm, and about as much CO_2 in the gas phase as in the experiments with cells. If $KHSO_4$ (which is not dissolved in H_2O in order to avoid pressure changes due to mixing of liquids) is then tipped into the trough, CO_2 is developed from the trough. This CO_2 is absorbed only physically by the water in the main compartment but chemically by the retaining fluid; the difference is the chemically bound CO_2, which, when divided by the increase in pressure and the fluid volume, gives the retention R. The method is to be preferred to the arrangement with the Siamese side-arms (*6,7*), because now the development of CO_2 occurs in the main compartment, and hence equilibrium is established more rapidly.

2. If the gas phase contains carbon monoxide, considerable amounts of CO are oxidized by the $KMnO_4$ dissolved in the trough under our experimental conditions, as Mr. Detlev Kayser has observed. The error caused by this oxidation is avoided by placing the $KMnO_4$ in the trough not in the dissolved state but as a solid, and by dissolving the $K_4Fe(CN)_6$ in 0.4 cc. rather than in 0.2 cc. H_2O. If the ferrocyanide is then placed in the trough at time t, the $KMnO_4$ is reduced only to the degree that it is dissolved and hence cannot oxidize any CO.

3. In experiments with hydrocyanic acid, hydrocyanic acid distills from the cell suspension into the trough when the contents of the trough and side-arm are mixed, but the pressure of the hydrocyanic acid developed thereby is so small that the manometry is not disturbed.

4. In experiments with *light*, it must be realized that ferrocyanide is oxidized to ferricyanide by light, and becomes alkaline. Consequently, we place the ferrocyanide not in the trough but in the side-arm, where it is generally not struck by light. If an experiment is done in diffuse light, the side-arm is wrapped in metal foil. If the $KMnO_4$ in the trough is

struck by light, it is warmed by absorption of light, especially since the trough is surrounded by air, which conducts heat away slowly. Here, therefore, in the transitions from darkness to light and from light to darkness, a thermomanometric effect occurs, which is eliminated by not making readings during the first 5 minutes after transitions.

REFERENCES

(1) KREBS, H. A.: Manometrische Messung des Kohlensäuregehaltes von Gasgemischen. Biochem. Ztschr. 220: 250–252, 1930.

(2) DICKENS, F., and SIMER, F.: Carbohydrate metabolism of normal and tumour tissue; method for measurement of respiratory quotient. Biochem. J. 24: 905–913, 1930.

(3) ———: Metabolism of normal and tumour tissue; method for measurement of respiratory quotient in serum. Biochem. J. 25: 973–984, 1931.

(4) ———: Metabolism of normal and tumour tissue; respiratory quotient in bicarbonate-media. Biochem. J. 25: 985–993, 1931.

(5) ———: Metabolism of normal and tumour tissue; note on carbon dioxide retention of serum: its use in measurement of tissue glycolysis. Biochem. J. 26: 90–98, 1932.

(6) WARBURG, O., and KRIPPAHL, G.: Ausbau der manometrischen Methoden. Liebigs Ann. 604: 94–96, 1957.

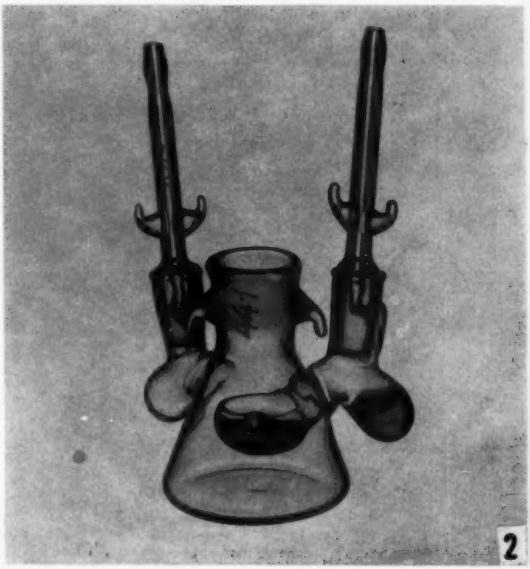
(7) ———: Weiterentwicklung der manometrischen Methoden. Ztschr. Naturforsch. 13b: 434–437, 1958.

PLATE 1

FIGURE 1.—Manometer vessel for determining the exchange of CO_2 and O_2 in one vessel. Volume about 20 cc.

FIGURE 2.—Manometer vessel for measuring the bound and the free carbon dioxide. Volume about 20 cc.

1


2

Differential Metabolic Responses of Susceptible and Resistant Mouse Leukemia Cells to 8-Azaguanine [1, 2]

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SUMMARY

Leukemia cells susceptible and resistant to 8-azaguanine have been studied with regard to direct metabolic changes in respiration and glycolysis produced by this drug. 8-Azaguanine caused a prompt and marked increase in glycolysis *in vitro* of susceptible cells but not of resistant cells, with concentrations of drug that ranged from about one-fourth to four times the pharmacologic level commonly employed (50 mg./kg.). Respiratory inhibition was observed in both types of cells, but at lower doses in the susceptible cells than in the resistant line. The increase in glycolysis and decrease in respiration were also observed *in vitro* following a single subcutaneous dose of 8-azaguanine given to mice bearing the susceptible cells. These metabolic effects observed *in vitro* tended to disappear after repeated daily exposure of the cells to the drug *in vivo*, and did so within a transplant generation. The significance of these findings is discussed in relation to a rationale of chemotherapy based on well-established metabolic properties characteristic of all cancer cells.—J. Nat. Cancer Inst. 24: 57–76, 1960.

8-AZAGUANINE, a close structural analogue of guanine (text-fig. 1), has been extensively investigated as an agent having activity against a variety of animal neoplasms (*1–4*). Clinical application of this compound (*5–7*) has been limited because of the severe dermatitis produced at doses considered inadequate for therapeutic effect.

When inhibition of growth of *Tetrahymena* by 8-azaguanine was first studied by Kidder *et al.* (*8*) in 1949, it was proposed that 8-azaguanine interfered with the utilization of guanine, a naturally occurring purine essential for growth. Effects of 8-azaguanine on mouse tumors were later found to be partially reversed by administration of guanine (*9*). Mandel reported (*10*) that whereas guanine was incorporated into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) of normal tissue, but not appreciably into that of tumor tissue, 8-azaguanine

[1] Received for publication August 28, 1959.

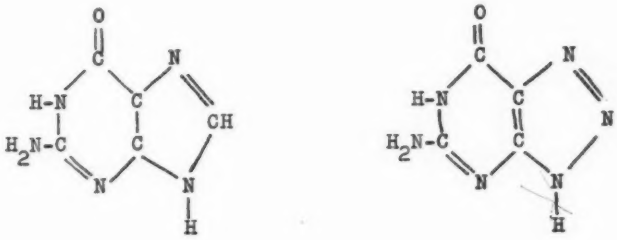
[2] The experimental work reported here was initiated at the suggestion of Dr. Howard Skipper, offered in a letter received from him dated January 6, 1958.

[3] Public Health Service Special Research Fellow of the National Cancer Institute.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

appeared to be selectively incorporated into tumor tissue when administered *in vivo*. At the same time, 8-azaguanine did not noticeably influence guanine metabolism in the intact animal. Mandel failed to observe any blocking of antitumor effects by guanine, and Gellhorn *et al.* (*11*) has even noted suggestive enhancement of 8-azaguanine inhibition of Brown-Pearce carcinoma by guanine.

Upon demonstration that a metabolic degradation product of 8-azaguanine was the inactive 8-azaxanthine, Hirschberg *et al.* (*12*) studied the guanase content of tumors sensitive and resistant to inhibition by 8-azaguanine. They reported a general correlation between low guanase content and tumor susceptibility on the one hand, and high guanase content and tumor resistance on the other hand, but these relationships did not hold in all instances.



Guanine 8-Azaguanine

TEXT-FIGURE 1.—Chemical formulas of guanine and 8-azaguanine.

Study of the mode of action of 8-azaguanine was facilitated in mice by the development by Law (*13*) of an 8-azaguanine-resistant mouse leukemic strain in ascitic form that had been derived from a susceptible parent line. Using these two strains, Davidson (*14*) demonstrated that C^{14}-labeled 8-azaguanine penetrated equally well into both the resistant and the susceptible cell types. Complementing this finding was the discovery by Brockman and coworkers (*15*, *16*) that susceptible tumors and bacteria metabolized 8-azaguanine to the corresponding ribotide, whereas resistant strains lacked this ability. Thus it was strongly suggested that the fraudulent nucleotide, 8-azaguanosine-5′-phosphate, was the biologically active material. With this demonstration of at least one metabolic alteration in the 8-azaguanine-resistant cells it became desirable to approach the question of the mechanism by which the fraudulent nucleotide might be cytotoxic to a malignant cell. The present studies were undertaken to investigate the effects of 8-azaguanine on the respiration and aerobic glycolysis of the two cell types, in the light of the possibility that the fraudulent nucleotide, if and when formed, might profoundly alter vital metabolic processes of cancer cells.

METHODS

(BALB/c × DBA/2)F_1 mice bearing L1210 leukemia in ascites form were used exclusively in these studies, and were kindly furnished by Dr. Lloyd Law of the National Cancer Institute. The original tumor line was susceptible to inhibition by 8-azaguanine, and the derived line had been made resistant by repeated exposure to the drug (*13*). The resistant strain had already been carried through 160 generations. Both tumors were inoculated intraperitoneally with approximately 10^3 cells, and both killed the host in 5 to 6 days. In these studies, cells were taken from mice at 3 to 5 days following inoculation. Cells were removed from the peritoneal cavity immediately after cervical disarticulation, and were diluted from ⅛ to 1/40 with Krebs-Ringer solution containing 2 mg. of glucose and 1.5 mg. of sodium bicarbonate per ml. fluid. This suspension was the usual experimental material. Centrifuged ascites serum was occasionally employed as diluting agent instead of Krebs-Ringer-bicarbonate solution. In each experiment from 1 to 3 mice were used from a given transplant group, and the ascitic fluid was pooled.

Analyses of cell metabolism were generally carried out by Summerson differential manometry (*17*, *18*) at 37° C., a technique for simultaneous determination of aerobic glycolysis ($Q_{N_2}^{O_2}$), oxygen consumption (Q_{O_2}), and respiratory carbon dioxide production (Q_{CO_2}) in one and the same vessel, with 5 percent CO_2/air as gas phase. Each Q value represents $mm.^3$ change per mg. dry weight per hour where 1 $mm.^3$ = 1/22,400,000 mole. The duration of experiments varied from 1 to 6 hours. Anaerobic glycolysis was measured in ordinary Warburg vessels in 5 percent CO_2/N_2.

In experiments dealing with the metabolic effects of 8-azaguanine administered *in vivo*, mice bearing 5- to 6-day-old L1210 ascites were given 250 to 400 mg. per kg. of 8-azaguanine subcutaneously. In such experiments, 1 or 2 control mice were given 1 ml. of water, and 1 to 2 treated mice were given 8-azaguanine in 1 ml. water, subcutaneously. Samples of ascitic fluid were removed from each animal for study prior to injection of drug or water, and at intervals thereafter. These intervals varied from 10 minutes to 3 hours. In presenting the data, 8 experiments were averaged, corrected for varying numbers of cells in each sample, and given as average of the values obtained before and after treatment. In these studies with treatment *in vivo*, cell metabolism was studied *in vitro* in Warburg vessels of equal size, under aerobic conditions only (5% CO_2/air as gas phase), and the manometric measurements obtained were expressed as net positive millimeters pressure change per mg. of dry weight of cells per hour. In such studies the pressure changes represent the algebraic sum of the processes of respiration and glycolysis, with the latter being responsible for the net positivity of all readings.

Glucose utilization was measured as the difference between glucose present before and after incubation. Glucose determinations were made by the glucose oxidase method (*19*), which has been found not to be affected by 8-azaguanine and ammonia (as well as guanine, guanosine,

hypoxanthine, and 8-azaxanthine) at concentrations involved in this study. Glucose consumption has been expressed as equivalents of lactic acid produced per mg. dry weight per hour, in order to be directly comparable with the manometric $Q_A^{O_2}$ values. Initial and final *p*H values were obtained, whenever possible, with a Beckman *p*H meter. Cell counts were made in duplicate in each experiment, in a Spencer Bright-line hemocytometer. Packed cell volumes were determined centrifugally in Bauer-Schenck hematocrit tubes. Dry weight was measured either directly or calculated on the basis of 8×10^6 leukemic cells = 6.6 $mm.^3$ volume = 1 mg. dry weight. In instances in which the relative number of red blood cells were small, the cell volume was preferred for calculation; when red cells were high, the cell count was employed. Red cells accounted for 10 to 50 percent of the cells in various experiments.

8-Azaguanine[5] was made up in a stock solution of 1 mg. per ml. after first dissolving in 0.5 N NaOH and adjusting to *p*H 7.5 with 0.1 N HCl. The drug was added to the manometer vessels to give final concentrations varying from 12.5 mg. per liter to 200 mg. per liter. The amount of drug commonly employed in antitumor studies *in vivo* is on the order of 50 mg. per kg. of mouse.

Errors.—Relative Q values in any given experiment are not subject to errors in estimation of dry weight, but absolute Q figures from one experiment to another are subject to the error in estimation of dry weight. In evaluating the data it should be borne in mind that, with the Summerson method, the most accurate determinations are Q_{O_2} and $Q_{A^2}^{O_2}$, less accuracy being possible with respect to Q_{CO_2}. The respiratory quotient ($RQ = Q_{CO_2}/Q_{O_2}$), and the ratio $Q_{A^2}^{O_2}/Q_{O_2}$ [an expression indicative of the degree of "malignancy" of cell metabolism (*18*)], both depend on two experimentally determined quotients and are thus subject to greater variability than their component parts, even within any one experiment, as well as between experiments.

To the extent that 8-azaguanine is deaminated by the cells, an error may be introduced in $Q_A^{O_2}$ as a result of retention of bicarbonate that leads to falsely low manometric values. We have found that deamination occurs in both the sensitive and resistant cell lines, and at 8-azaguanine concentrations of 100 and 200 mg. per liter deamination may become large enough to yield slightly low values for manometric aerobic glycolysis. Although measurements of aerobic glycolysis by glucose consumption are not affected by CO_2 retention, they have the disadvantage of involving differences that may at times be small compared to the initial glucose content, whereas manometric determinations of aerobic glycolysis give pressure changes without necessary reference to the absolute quantity of glucose initially present.

[5] Kindly furnished by Dr. Jack Davidson of the National Cancer Institute.

RESULTS

8-Azaguanine-Susceptible Strain

Representative metabolic studies *in vitro* with the 8-azaguanine-sensitive line under aerobic conditions are summarized in table 1. In experiment 1, control quotients for these cells were measured in triplicate, with good agreement; the average $Q_A^{O_2}$ was 14.2. With 12 mg. per liter 8-azaguanine $Q_A^{O_2}$ was increased to 20.9, with 25 mg. per liter to 25.2, and with 50 mg. per liter to 27.6, corresponding to increases of 47, 78, and 95 percent respectively. This pattern of glycolytic stimulation was confirmed by measuring glucose consumption expressed as lactic acid equivalent. Glycolytic stimulation was noted as soon as satisfactory manometric readings could be obtained after equilibration on the bath. In experiment 1, Q_{O_2} decreased from 12.2 in the controls to 9.3 at 50 mg. per liter of the drug. This respiratory inhibition was observed at lower concentrations of the drug than in some other experiments. Q_{CO_2} decreased almost in proportion to Q_{O_2}, resulting thereby in only a small change in RQ. The ratio $Q_A^{O_2}/Q_{O_2}$ was increased by the drug 2.5-fold (from 1.2 to 3.0). All effects in this experiment may be attributed to concentrations of 8-azaguanine usually considered within the chemotherapeutic range. The word "stimulation" is used throughout this paper broadly and conventionally, with no attempt to distinguish between (a) an improved preservation of observed rates falling with time or (b) an absolute increase in rates believed (by extrapolation) to prevail before actual commencement of measurements *in vitro* (*i.e.*, prior to experimental $t = 0$).

In experiment 2 with the susceptible strain the average control $Q_A^{O_2}$ was 12.7. When 25 mg. per liter were added *in vitro* the aerobic glycolysis increased to 19.2, and reached a maximum of 20.7 with 50 mg. per liter. It is interesting to note that the $Q_A^{O_2}$ was essentially unchanged over the concentration range 50 to 200 mg. per liter of 8-azaguanine. However, there is an apparent discrepancy in the lactic acid equivalents of glucose as this continued to increase from 23 at 50 mg. per liter to 31 at 200 mg. per liter. At 100 to 200 mg. per liter, ammonia production was appreciable, as found by independent Nessler's reagent determinations, and amount of base formed as NH_3 corresponded well quantitatively with the lowering in manometric result. In this experiment the Q_{O_2} did not begin to decrease until 8-azaguanine concentrations of greater than 50 mg. per liter were used, and then it fell from 11.7 in the controls to 7.8 at 200 mg. per liter. Thus, in this study, significant glycolytic stimulation was noted at 25 to 50 mg. per liter without any change (decrease) in Q_{O_2}. The Q_{CO_2} decreased roughly in proportion to the Q_{O_2}, so that there was no major change in the RQ. The metabolic ratio, $Q_A^{O_2}/Q_{O_2}$, increased from 1.1 in the controls to 2.6 at 200 mg. per liter.

Experiment 3 again illustrates glycolytic stimulation at 25 mg. per liter, with maximum stimulation achieved at 100 mg. per liter. In this study, Q_{O_2} was only inhibited at 200 mg. per liter, and in fact an initial slight

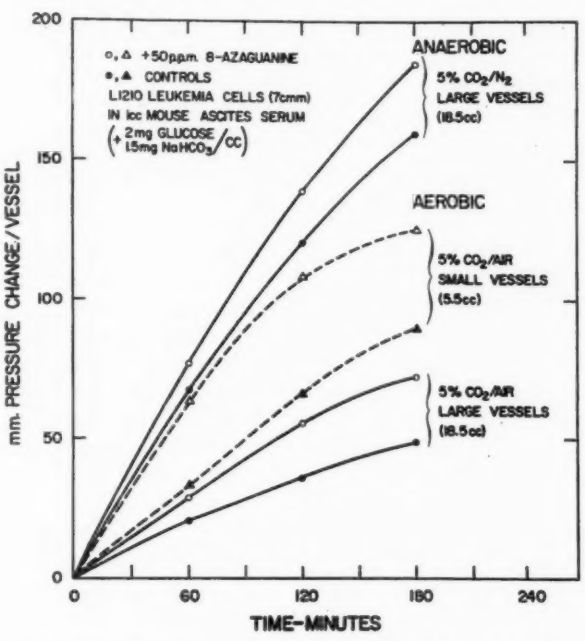
TABLE 1.—Metabolic effects of 8-azaguanine on L1210 (sensitive) cells *in vitro*

Experiment No.	Date 1958	Hours	Concentration of 8-azaguanine (*mg./l., ppm*)	Aerobic glycolysis $Q_{La}^{O_2}$	Oxygen consumption Q_{O_2}	Respiratory CO_2 production Q_{CO_2}	Respiratory quotient (RQ)	Glycolysis: Respiration $Q_{La}^{O_2}/Q_{O_2}$	Lactic acid equivalent $Q_{Glucose}$
1	2/11	2	0	14.7	12.0	9.4	0.78	1.2	20
			0	14.7	12.2	9.8	0.81	1.2	20
			0	12.9	12.4	11.4	0.92	1.0	16
			Average	14.2	12.2	10.2	0.84	1.2	19
		2	12	20.9	11.1	8.4	0.76	1.9	23
		1	25	25.2	11.0	9.2	0.83	2.3	31
		2	50	27.6	9.3	7.2	0.78	3.0	33
2	2/19	3	0 (dupl.)	12.7	11.7	9.3	0.80	1.1	13
			25	19.2	10.7	—	—	1.8	19
			50	20.7	11.0	8.0	0.73	1.9	23
			100	19.1	8.2	6.9	0.84	2.3	24
			200	20.5	7.8	6.5	0.83	2.6	31
3	3/4	3	0	16.2	13.3	10.0	0.89	1.2	
			25	23.2	15.4	13.2	0.86	1.5	
			50	23.2	14.0	10.2	0.73	1.7	
			100	24.9	14.6	11.0	0.75	1.7	
			200 (dupl.)	20.8	9.6	7.0	0.75	2.1	
Average (controls)			0	14.5	12.4	9.6	0.8	1.2	

stimulation was suggested at lower concentrations. In this experiment the RQ decreased from 0.89 in the control to approximately 0.75 for 50, 100, and 200 mg. per liter. Again, the ratio, $Q_A^{O_2}/Q_{O_2}$, increased with increasing 8-azaguanine concentration.

Some half dozen other studies carried out with the susceptible cell line, not detailed here, showed glycolytic stimulation as high as 100 percent at concentrations of 50 to 100 mg. per liter.

Text-figure 2 shows one of our earliest survey experiments, performed in mouse ascitic serum, both anaerobically and aerobically, in vessels of different sizes, and as a function of time. In this survey experiment, we were not interested in obtaining absolute Q values but merely in looking qualitatively to find any manometric effects that might be induced by application of 8-azaguanine. It will be seen that anaerobically as well as aerobically the pressure changes progressively *increased* with time, upon treatment of the sensitive cells with 8-azaguanine, which had been added at $t = -20$ minutes, just before equilibration of the vessels on the thermostated bath. As would be expected on methodological grounds, the relative pressure differences were greater in the smaller (aerobic) vessels. Presumably the absolute $Q_A^{O_2}$ values were the same in both small and large vessels except as influenced by 8-azaguanine. The large Pasteur effect (reduction of glycolysis by O_2) typical of cancer cells is to be seen in the



TEXT-FIGURE 2.—Effect of 8-azaguanine on anaerobic and aerobic pressure changes (glycolysis) produced by susceptible L1210 leukemia cells in mouse ascitic serum as a function of time.

much smaller positive pressures obtained aerobically than anaerobically in the vessels of identical size (18.5 ml.), either in the presence or absence of 8-azaguanine. If there had been no glycolysis, the pressure changes would, of course, have been negative, as a result of respiration alone, so that the base lines for *total* positive pressure changes due to glycolysis alone lie somewhere below zero on the ordinate in text-figure 2, and differently so for the different conditions. Since respiration is, if anything, decreased by 8-azaguanine, the effects of this drug on glycolysis *per se* were, if anything, greater than indicated in text-figure 2, after due algebraic correction for such base-line shifts.

8-Azaguanine-Resistant Strain

Results obtained with the 8-azaguanine-resistant cell line are summarized in table 2. In experiment 1, the average $Q_A^{O_2}$ was 17.9, and with increasing concentrations of 8-azaguanine it was 18.0, 15.7, and 16.8, all probably within the experimental error, although a slight decrease (rather than the increase observed in the susceptible line) is indicated. At concentrations up to 100 mg. per liter, the variations in Q_{O_2} were not significant. The RQ did decrease from a control value of 0.64, to 0.51 at the highest concentration of 8-azaguanine, but the ratio, $Q_A^{O_2}/Q_{O_2}$, and the glucose-lactic acid equivalents did not change. In experiment 2, at the lower concentration of 8-azaguanine, there was also no effect on respiration or glycolysis.

In experiment 3 there was no effect of 8-azaguanine on glycolysis at concentrations up to 200 mg. per liter. The Q_{O_2} of 22.5 was unusually high in this study and was not changed by 8-azaguanine even at the maximum concentration. Although the aerobic glycolysis is well within the range seen with malignant tumors, a Q_{O_2} of the magnitude of 22.5 is rarely seen with malignant tissues—especially in the medium employed. This peculiarity is also reflected by $Q_A^{O_2}/Q_{O_2}$ being well below unity, as commonly seen with benign tumors or some normal tissues.

The fourth-listed experiment on the 8-azaguanine-resistant cell population indicates some glycolytic inhibition at concentrations of 100 mg. per liter and 200 mg. per liter, that does not seem referable entirely to NH_3 formation. The latter concentration decreased the observed $Q_A^{O_2}$ from control levels of 19.6 to 11.1, the former concentration to 16.8. Glycolytic inhibition was also found in experiments 1 and 3 if the figures are taken literally, although by themselves they were not considered as significant changes. The suggestive decrease in glycolysis at the higher concentrations of 8-azaguanine in the resistant cell line stands in contrast to the definite stimulation noted in table 1 with the sensitive cell line. Q_{O_2} was inhibited only at 200 mg. per liter and not at lower concentrations.

To compare the metabolic rates in the two cell lines without addition of 8-azaguanine the control Q values of each experiment were averaged. Aerobic glycolysis averaged 18.4 in the resistant group and 14.5 in the sensitive; Q_{O_2} was 14.9 in the resistant and 12.4 in the sensitive strain.

TABLE 2.—Metabolic effects of 8-azaguanine on L1210 (resistant) cells *in vitro*

Experiment No.	Date 1958	Hours	Concentration of 8-azaguanine (*mg./l., ppm*)	Aerobic glycolysis $Q_{A}^{O_2}$	Oxygen consumption Q_{O_2}	Respiratory CO_2 production Q_{CO_2}	Respiratory quotient (*RQ*)	Glycolysis: Respiration $Q_{A}^{O_2}/Q_{O_2}$	Lactic acid equivalent $Q_{Glucose}$
1	2/13	3	0	18.0	12.1	7.3	0.60	1.5	16
			0	18.8	13.3	8.1	0.60	1.4	19
			0	16.8	12.6	9.0	0.71	1.3	16
			Average	17.9	12.7	8.1	0.64	1.4	17
			25	18.0	12.6	7.0	0.56	1.4	16
			50	15.7	11.3	7.7	0.68	1.4	
			100	16.8	13.2	6.7	0.51	1.3	16
2	2/13	2	0	21.1	14.1	10.2	0.72	1.6	
			12	20.4	13.8	8.8	0.64	1.5	
3	2/25	4	0	15.2	21.7	17.6	0.81	0.70	16
			0	15.4	22.8	18.1	0.81	0.67	16
			0	14.8	22.7	19.4	0.85	0.65	17
			Average	15.2	22.5	18.4	0.82	0.67	16
			50	15.3	22.7	19.4	0.85	0.67	17
			100	15.1	23.4	20.1	0.86	0.65	19
			200	14.1	22.6	20.1	0.89	0.63	
4	3/7	4	0 (dupl.)	19.6	10.5	6.3	0.60	1.9	
			50	19.1	9.7	5.2	0.54	2.0	
			100	16.8	10.2	6.4	0.62	1.7	
			200 (dupl.)	11.1	8.4	6.3	0.75	1.3	
Average (controls)			0	18.4	14.9	10.8	0.7	1.2	

The RQ of the resistant line averaged 0.7, that of the sensitive line 0.8, and $Q_A^{N_2}/Q_{O_2}$ was 1.2 for both types, in the absence of 8-azaguanine.

Administration of 8-Azaguanine *In Vivo*

Since interference of aerobic as well as anaerobic glycolysis was observed, when susceptible L1210 ascites cells were incubated with 8-azaguanine *in vitro*, we studied the effects of drug administration *in vivo*. Table 3 gives the average values obtained in 8 experiments in which mice bearing the L1210 susceptible strain were treated with 8-azaguanine *in vivo*. A portion of each ascites suspension removed was also further treated with 8-azaguanine *in vitro*. Thus, initial and final samples of the control and treatment groups were also incubated with 8-azaguanine *in vitro*. Whereas cells taken from the control group liberated 37 percent less net positive pressure in the final sample compared to the initial sample, cells taken from the group treated *in vivo* with 8-azaguanine were found to liberate 36 percent more net positive pressure in the final sample. In the portion of the experiment *in vitro* it was found that cells from the control group had the same sensitivity to 8-azaguanine *in vitro* in both the initial and final samples, namely, 36 and 34 percent net increase in positivity respectively. In the case of the group treated with 8-azaguanine *in vivo*, the responsiveness *in vitro* was significantly less in the final sample as compared with the initial sample (plus 20% *versus* plus 46% in the initial portion). Similar experiments were also made with Summerson differential manometers, and in these it was demonstrated that the net increase in pressure that occurs in L1210 cells taken from 8-azaguanine-treated mice is due to an increase in aerobic glycolysis together with a smaller inhibition of respiration, much the same as the changes occurring in the susceptible line treated with 8-azaguanine *in vitro* only, as was previously described.

The type of experimentation *in vivo* described is *a priori* obviously subject to greater variability and irregularity of result than the type *in vitro*

TABLE 3.—Metabolic effects of 8-azaguanine (*in vivo* and *in vitro*) on susceptible L1210 cells*

Treatment of mice	$t=0$ control sample *(removed before S.C. 8-azaguanine)*		Cells removed from mouse 1 to 2 hours following S.C. 8-azaguanine	
	No addition *in vitro*	8-Azaguanine added *in vitro*	No addition *in vitro*	8-Azaguanine added *in vitro*
Mice treated with 300 mg. per kg. S.C. after $t=0$ control sample was taken	17.3	25.2 (+46%)	23.5 (+36%)	28.2 (+20%)
Controls given 1 cc. H_2O S.C. after $t=0$ control sample was taken	19.9	27.0 (+36%)	14.5 (−37%)	19.3 (+34%)

*Results expressed as mm. pressure change per mg. dry weight per hour, for 2d to 3d hour. Each value is an average of 8 experiments.

because of the more complicated procedures and treatment of host mice involved, so that the values reported in table 3 are indicative of the average trends, from which individual experiments may vary considerably with respect to details, and even in regard to sign. For present purposes a statistical treatment, with additional experimentation to obtain Chi-square test analysis, did not seem warranted, especially since the data are reported not in terms of cubic millimeters of glycolysis but merely pressure changes. The quantitative percentage effects reported in table 3 for the 2d and 3d hour *in vitro* would appear smaller if values for the 1st hour were included, since the development of 8-azaguanine action is measurably progressive with time.

With varied doses of 8-azaguanine it was found that a single subcutaneous dose of 300 mg. per kg. *in vivo* gave metabolic alterations similar to those found on incubation *in vitro* with 50 mg. per liter (table 4). The above results indicated that cells removed at an advanced stage of the disease were still susceptible to the metabolic effects of 8-azaguanine after administration *in vivo*.

TABLE 4.—Metabolic effects of 8-azaguanine given *in vivo* to susceptible L1210 mouse leukemia ascites cells and examined *in vitro* after 3 hours*

Treatment of mice	Control	*In vivo*	(*S.C.*)	Control (*in vitro*)
8-Azaguanine (*mg./kg.*)	0	150	300	50
$Q_{A}^{L^2}$ acid production	12. 2	13. 1	16. 3	17. 8
Q_{O_2} oxygen consumption	13. 0	12. 0	8. 8	10. 6
$Q_{A}^{L^2}/Q_{O_2}$	0. 9	1. 1	1. 9	1. 7

*Q=mm.³/mg. dry weight per hour; Summerson manometry.

To test the response of susceptible cells repeatedly exposed *in vivo* to 8-azaguanine, mice bearing susceptible cells inoculated intraperitoneally were treated daily with subcutaneous injections of 8-azaguanine at 75 mg. per kg. beginning with the day following inoculation. The animals were killed on the 9th day at the terminal stage of the disease and after 8 injections. The rapidly proliferating, now somewhat biologically resistant, "susceptible" cells were studied in Summerson manometers with respect to their response to 8-azaguanine *in vitro*, treatment *in vivo* having been stopped 24 hours earlier. The results are summarized in table 5

TABLE 5.—Incubation *in vitro* of susceptible L1210 leukemic cells with 8-azaguanine—after daily exposure *in vivo* to the drug*

8-Azaguanine (*mg./l.*)	Control (*average*) 0	25	50	100	200
$Q_{A}^{L^2}$	5. 4	5. 2	6. 7	5. 7	6. 0
Q_{O_2}	11. 1	11. 3	9. 6	12. 0	10. 8
Q_{CO_2}	8. 3	8. 6	7. 0	9. 1	8. 3
RQ	. 74	. 76	. 73	. 75	. 77
$Q_{A}^{L^2}/Q_{O_2}$	. 49	. 45	. 71	. 48	. 55

*Injections of 8-azaguanine (75 mg./kg.) were begun the day following inoculation and continued for 8 injections. Animals were killed on the 9th day after tumor implantation.

GLUCOSE + ADENOSINETRIPHOSPHATE (ATP)	HEXOKINASE (MITOCHONDRIAL) → (INSULIN:ANTI-INSULIN REGULATED)	GLUCOSE-6-PHOSPHATE + ADP
INHIBITORS	*INHIBITORS*	*INHIBITORS*
2-DEOXY-GLUCOSE 2-DEOXY-GALACTOSE GALACTOSE 6-FLUORO-GLUCOSE	(PURINES) 6-MERCAPTOPURINE ADENINE ADENOSINE ADENOSINEMONOPHOSPHATE ADENOSINEDIPHOSPHATE (PYRIMIDINES) 5-FLUOROURACIL 5-FLUOROURIDINE 5-FLUORO-2′-DEOXYURIDINE 5-FLUOROCYTIDINE 5-FLUORO-2′-DEOXYCYTIDINE URIDINE 6-AZAURACIL 6-AZATHYMINE (FOLICS) AMETHOPTERIN (METHOTREXATE) FOLIC ACID (PTEROYLGLUTAMIC ACID) TEROPTERIN (PTEROYLTRIGLUTAMIC ACID)	(STEROIDS) CORTISONE HYDROCORTISONE DEOXYCORTICOSTERONE PREDNISOLONE TESTOSTERONE PROGESTERONE DIETHYLSTILBESTROL (PITUITARY FACTOR) ANTI-INSULIN LIPOPROTEIN (*PODOPHYLLUM* COMPOUNDS) PODOPHYLLOTOXIN PELTATINS NCI-3022 (ANTIBIOTICS) PENICHROMIN
	STIMULATORS 8-AZAGUANINE	*STIMULATORS* INSULIN ENDOTOXIC POLYSACCHARIDES

TEXT-FIGURE 3.—Proposed primary sites of chemical action of several cancer metabolic inhibitors and stimulators involving the hexokinase reaction in various tumors under various conditions [*cf.* (*21*)].

drug. Mandel (*10*) and Graff *et al.* (*26*) have shown that in tumors the action of 8-azaguanine is not due to a simple antimetabolite relationship with guanine. Mandel found (*27*) that growth inhibition of *Bacillus cereus* by 8-azaguanine occurred within minutes after addition of the drug, and before any measurable amount of drug was incorporated into DNA, and relatively very little into RNA. Conversely, after large amounts of 8-azaguanine had become incorporated into cell RNA, no growth inhibition occurred when such cells were placed in a normal medium free of 8-azaguanine. Here the antitumor effects of 8-azaguanine or its ribotide are not readily explained on the basis of interference of, or fraudulent, nucleic acid synthesis. It is of interest in this connection that Sartorelli *et al.* (*28*) found that both thioguanine resistant and susceptible sublines of Ehrlich ascites cells appeared to show equal capacity to convert thioguanine to its nucleotide form over a considerable range of therapeutic concentrations.

Finkelstein *et al.* (*29*) studied the oxygen consumption of various tissues incubated with 8-azaguanine *in vitro*. They did not find any effect on oxygen uptake in slices of Sarcoma 37, liver, spleen, or kidney. When given *in vivo* at doses of 200 mg. per kg. or larger, a respiratory inhibition was then found in the tumor slice, presumably associated with hemorrhage and softening of the tumor. Glycolysis was not measured.

The present studies show that another qualitative difference between resistant and susceptible leukemic cells lies in the glycolytic interference produced in the latter by 8-azaguanine. This has been found not only with drug addition *in vitro*, but also after administration *in vivo*. This metabolic effect tends to disappear after continued daily exposure *in vivo* of the susceptible cells to 8-azaguanine. Whether the glycolytic stimulation is due to 8-azaguanine or to 8-azaguanosine-5′-phosphate remains to be tested when sufficient amounts of the latter become available. Preliminary experiments with Hobby (*30*), in our laboratory, have demonstrated marked glycolytic stimulation with 8-azaguanine in mouse melanoma mitochondria, a system which would scarcely be expected to be capable of synthesizing the ribotide—at least under the conditions employed. The results obtained with melanoma mitochondria could be due to a counteraction of destruction of adenosinetriphosphate, since restrainers of adenosinetriphosphatase have been found to counteract glycolytic decay in melanoma homogenates. Similar phenomena may occur with L1210 cells treated with 8-azaguanine *in vitro*. The enhancement *in vitro* of aerobic glycolysis of L1210 cells by 8-azaguanine appears to involve in part a counteraction of the rate of decay of glycolysis with time, and Ehrlich ascites cells show a similar response to 8-azaguanine. Adenine gave marked counteractions of glycolytic decay in not only L1210-8-azaguanine-susceptible but also L1210-8-azaguanine-resistant cells, as well as in Ehrlich ascites cells. 8-Azaxanthine did not counteract glycolytic decay in any of these cell lines.

TEXT-FIGURE 3.—Proposed primary sites of chemical action of several cancer [illegible] reaction in various tumors under various conditions [*cf.* (*21*)].

REFLECTIONS

As already indicated, our experimental studies of the past few years have brought to light the fact that the majority of types of cancer chemotherapeutic agents employed clinically today turn out, regardless of original rationale of derivation, or discovery, to have powerful and immediate effects on one or both of the two primary, over-all energy-providing processes of cancer-cell metabolism, namely, mainly glycolysis and not infrequently respiration. Such primary energy effects, which may at times be accompanied by nucleic acid derangements, can result in many secondary chemical and biological effects including derangement of coenzyme syntheses, general cell breakdown, or death of the cancer cell. Furthermore, although various types of cancer cells are affected unequally by different agents, as might be anticipated, there is good general correlation between clinical responses to, and metabolic effects of, a given agent. This is true with respect to both positive and negative clinical responses. Thus, for example, methotrexate will produce both clinical responses and cellular glycolytic inhibitions in many cases of human acute lymphatic leukemia, but relatively rarely in human acute myelogenous leukemia. Myleran, frequently employed in chronic myelogenous leukemia, produced marked respiratory inhibition of leukemic cells taken from circulating blood. 2-Deoxy-D-glucose, found by a number of workers to affect animal tumor growth with favorable chemotherapeutic index, produced marked glycolytic inhibitions in every type of cancer cell we have studied, including mouse melanomas, leukemias, and ascites cells, and a wide variety of types of human leukemic cells. Commonly employed compounds already listed all produced notable glycolytic inhibitions or stimulations (departure from metabolic homeostasis) in a wide variety of cancer cells tested, especially where glycolysis was under marked insulin: anti-insulin regulation at the (mitochondrial) hexokinase level (*21, 31–35;* text-fig. 3).

The notable quantitative and qualitative variability of action of chemotherapeutic agents, observed both clinically *in vivo* and metabolically *in vitro*, appears to depend, indeed, in great part on the degree of insulin: anti-insulin regulation of fermentation at its initial step, namely, the hexokinase reaction whereby glucose is phosphorylated with adenosinetriphosphate. This regulation (*21, 32–35*) has been observed to increase among a spectrum of tissues in the following approximate order: mouse ascites carcinomas, most tumor tissue-culture lines, human acute and chronic myelogenous leukemias, human chronic and acute lymphatic leukemias, Gardner mouse lymphosarcoma, mouse amelanotic melanoma S91A, mouse mammary tumors, mouse melanoma S91, normal tissues (*e.g.*, adipose, diaphragm, embryonic, kidney, lung, liver, diabetic). The degree of regulation and the spectrum order are further affected by a variety of factors, including temperature, concentration of glucose and insulin, substitution of fructose for glucose, sodium/potassium ratio, and stress. We conclude that metabolic analysis along the lines adumbrated offers one of the most constructive, promising, rapid, and new approaches to chemotherapeutic screening and the selection of agents for clinical trial or treatment.

To date, the great bulk of studies on cancer chemotherapy has been concerned with the chemical synthesis or isolation, animal screening, and clinical testing of tens of thousands of substances, especially antimetabolites, and for the most part singly rather than in combinations. The majority of such studies have been empirical in the sense that they have been based mainly on the hope or the gambler's expectation that eventually some favorable difference in response between cancer and normal cells will be found with at least one or more cancers. Animal and clinical testing has often been carried out with little regard to the known characteristic metabolic nature of the cancer cell itself. This largely sheer gamble at fantastic odds now being undertaken empirically in the field of cancer chemotherapy by and large, *with mice, men, and money* could well lead, in the next 5 to 15 years, to overwhelming disappointment, unless the pendulum of effort is swung back to a better balance of less gamble and still more basic approach. Continued reliance mainly on the seeming shortcut of the gambler's expectation will never suffice for a broad

all-out solution of the medical problem of cancer, which medical problem is the chief reason for studying cancer at all.

The concept that nucleic acids occupy a key position in the economy of the cell has led to numerous attempts to control growth and multiplication by interference with their synthesis in cancer cells. Thus, certain antimetabolites have been aimed chiefly against specific synthetic steps in purine syntheses *in vivo*, on the chance that they might act as anticancer agents by altering the chemical structures of nucleic acids or related coenzymes formed. Although in instances such chemical structures have indeed been so altered, in both cancer and normal cells, no patterns of alteration characteristic for each of these classes of cell types have yet been established or even indicated. Furthermore, none of these chemotherapeutic materials employed has shown widespread applicability to individuals with cancer, and even within a single disease, such as acute lymphatic leukemia, the response of a particular patient to a given antimetabolite has not been generally predictable. Where clinical chemotherapeutic effects have been observed, it is still highly questionable whether the *primary* mechanism of inhibition involves disturbed chemical structures of nucleic acids or related enzymes, as widely believed. Current cancer chemotherapy still remains largely an "alchemotherapy," a grand collection of unguided missiles with no central biochemical target *common to cancer cells generally*.

It is remarkable that little or no use has been made of a rationale of cancer chemotherapy based on factual differences *already established* to exist between cancer and normal cells generally. In particular, little use has been made of the fact, first discovered by Otto Warburg in the twenties, and now universally accepted, that all cancer cells without exception produce large amounts of lactic acid from glucose in the presence of oxygen gas (aerobic fermentation). Expressed more biologically, all cancer cells growing in the presence of air lead a "partially anaerobic life," obtaining their energy, as Pasteur showed a century ago with yeast, partly from respiration and partly from fermentation. This partially anaerobic life of cancer cells that takes place in the presence of oxygen gas is not to be confused with the facultative anaerobic life of microorganisms that may take place in the absence of oxygen gas.

The property of aerobic fermentation may be regarded as the only qualitative biochemical lesion that has yet been found to be an invariable, experimental characteristic of all living cancer cells studied. Moreover, in no other field of science that has been applied to the problem of cancer (*e.g.*, pathology, histology, cytology, etiology, genetics, radiology) has there yet been discovered so universal a lesion, and hence so universal a potential point of chemotherapeutic attack applicable to not only just some, but in principle, all living cancer cells.

Aerobic fermentation in cancer cells may be termed a biochemical lesion because, by contrast, few—if any—normal, living animal cells have shown unmistakably a large aerobic fermentation under conditions *in vivo*, or conditions equivalent thereto. The distinction between living cancer cells and living normal cells in respect to aerobic fermentation *in vivo* is quantitatively so great as to be, in effect, virtually qualitative. *This is the first law of cancer biochemistry.* [It is only when normal cells are dying, are harmed *in vitro* (as by unsuitable media), or are deliberately broken up (homogenized) that they may sometimes show an aerobic fermentation quantitatively approaching that of living cancer cells.] In any event, the chemotherapeutic importance of any possible exceptions to this generalization still remains to be discovered.

Until quite recently, the exact enzymatic locus of the biochemical mechanism governing the enormous fermentation rate of cancer cells compared to normal cells has been one of the profoundly mysterious, unsolved problems of cancer metabolism. Our studies of the last few years (*31–35*) have demonstrated that, of the 12 or more enzymes involved in fermentation, the controlling enzymatic locus lies at the very gateway of glucose metabolism, namely, at the initial phosphorylation of glucose in the mitochondrial hexokinase enzyme reaction, as illustrated in text-figure 3. A high rate of this glucose phosphorylation is a demonstrated *sine qua non* for cancer metabolism. In cancer cells a decreased restraint, or decreased inhibition, of the

hexokinase reaction by the anti-insulin hormones (*e.g.*, types listed in the third column of text-fig. 3) is chiefly responsible for permitting the high fermentation (*30–35*). *This is the second law of cancer biochemistry.* Conversely, in normal cells, extensive anti-insulin hormonal inhibition of the hexokinase reaction accounts for the absence of fermentation. In cancer cells an important hormonal brake has been weakened or eliminated, and this is, or may become, their Achilles' heel, their (to shift from Greek to German mythology) Siegfried's linden leaf (*21*). It may not be possible to discover a single practical chemical agent which is capable of destroying most types of cancer cells. However, the rational exploitation of the metabolic lesion which characterizes all cancers, *i.e.*, their abnormally high glycolytic capacity, may make possible therapeutic results far beyond those obtainable with a single agent. With such a rational exploitation of this "central target," characteristic of cancer cells generally, we can envision synergistic unions between such clinical disciplines as chemotherapy, radiology, and serology.

Enhanced, additive, or synergistic inhibitions have been observed when agents from two or more types of inhibitors (substrate, coenzyme, or enzyme) have been employed simultaneously. The possibility of multiple inhibition of the hexokinase reaction, at three different sites, is thus an important feature of the proposed rationale of cancer chemotherapy, especially in connection with overcoming the development of resistant strains of cancer cells. The success now achieved in the treatment of tuberculosis by multiple chemotherapy (*e.g.*, with isonicotinic acid hydrazide, thiocarbazones, and streptomycin, as first worked out by Gerhard Domagk) stands as a notable and proven example of overcoming the problem of acquired resistance. This example also provides a biochemical moral, when it is realized that the most prominent agent, isonicotinic acid hydrazide, represents a chemotherapeutic development based on presumed competitive action against a prominent, naturally occurring coenzyme (or coenzymes) containing nicotinic amide. In cancer one has the added advantage that another equally prominent coenzyme, adenosinetriphosphate, is now already definitely established to be a central point of chemotherapeutic attack (text-fig. 3).

We may recapitulate the foregoing reflections by citing the following authoritative and optimistic statement by a man who has worked on cancer longer than anyone, for over 50 years, and who is still working in the field with undiminished interest and vigor. He says,

> The fact that cancer cells are partial anaerobics, in contrast to normal embryonic cells that are pure aerobics, is closely related to the work of Pasteur, who discovered anaerobic life, and at once knew its significance. It was Pasteur who discovered differentiation by respiration and dedifferentiation by fermentation—by an experiment that stands at the head of all biochemistry of cancer. Certainly nothing could be more in the spirit of Pasteur than to build the future chemotherapy of cancer upon the partial anaerobiosis of cancer cells. It is my firm conviction that then cancer chemotherapy will be successful.—Otto Warburg, July 1957.

REFERENCES

(*1*) Gellhorn, A., Engelman, M., Shapiro, D., Graff, S., and Gillespie, H.: The effect of 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine (guanazolo) on a variety of neoplasms in experimental animals. Cancer Res. 10: 170–177, 1950.

(*2*) Law, L. W.: Studies on the effects of a guanine analog on acute lymphoid leukemias of mice. Cancer Res. 10: 186–190, 1950.

(*3*) Sugiura, K., Hitchings, G. H., Cavalieri, L. F., and Stock, C. C.: The effect of 8-azaguanine on the growth of carcinoma, sarcoma, osteogenic sarcoma, lymphosarcoma and melanoma in animals. Cancer Res. 10: 178–185, 1950.

(4) FINKELSTEIN, M., and THOMAS, P. A.: Studies on the effect of 8-azaguanine on sarcoma 37 in mice. Cancer Res. 11: 801–806, 1951.

(5) COLSKY, J., GREENSPAN, E. M., and SCHOENBACH, E. B.: Observations on the effects of administration of guanazolo in patients with disseminated neoplasms. Cancer 5: 1221-1224, 1952.

(6) COLSKY, J., MEISELAS, L. E., ROSEN, S. J., and SCHULMAN, I.: Response of patients with leukemia to 8-azaguanine. Blood 10: 482–492, 1955.

(7) PARKS, R. E., JR.: Antimetabolite studies in *Tetrahymena* and tumors. *In* Antimetabolites and Cancer (Symposium of AAAS). 1953, pp. 175–197.

(8) KIDDER, G. W., DEWEY, V. C., PARKS, R. E., JR., and WOODSIDE, G. L.: Purine metabolism in Tetrahymena and its relation to malignant cells in mice. Science 109: 511–514, 1949.

(9) ———: Further evidence on the mode of action of 8-azaguanine (guanazolo) in tumor inhibition. Cancer Res. 11: 204–211, 1951.

(10) MANDEL, H. G.: Some aspects of the metabolism of 8-azaguanine. *In* Antimetabolites and Cancer (Symposium of AAAS). 1953, pp. 199–218.

(11) GELLHORN, A., HIRSCHBERG, E., and KELLS, A.: The effect of purines, nucleosides, and nucleotides on the carcinostatic action of 8-azaguanine. J. Nat. Cancer Inst. 14: 935–939, 1954.

(12) HIRSCHBERG, E., KREAM, J., and GELLHORN, A.: Enzymatic deamination of 8-azaguanine in normal and neoplastic tissues. Cancer Res. 12: 524–528, 1952.

(13) LAW, L. W.: Resistance in leukemic cells to a guanine analog, 8-azaguanine. Proc. Soc. Exper. Biol. & Med. 78: 499–502, 1951.

(14) DAVIDSON, J. D.: Permeability of resistant L1210 leukemia cells to 8-azaguanine and 6-mercaptopurine. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 290–291, 1958.

(15) BROCKMAN, R. W., SKIPPER, H. E., and THOMPSON, J. R.: Comparison of the metabolism of 8-azaguanine *in vivo* by drug-susceptible and drug-resistant experimental neoplasms. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 284, 1958.

(16) BROCKMAN, R. W., SPARKS, C., HUTCHISON, D. J., and SKIPPER, H. E.: A mechanism of resistance to 8-azaguanine. I. Microbiological studies on the metabolism of purines and 8-azapurines. Cancer Res. 19: 177–188, 1959.

(17) SUMMERSON, W. H.: A combination simple manometer and constant volume differential manometer for studies in metabolism. J. Biol. Chem. 131: 579–595, 1939.

(18) BURK, D., SPRINCE, H., SPANGLER, J. M., KABAT, E. A., FURTH, J., and CLAUDE, A.: The metabolism of chicken tumors. J. Nat. Cancer Inst. 2: 201–240, 1941.

(19) KESTON, A. S.: Specific colorimetric enzymatic analytical reagents for glucose. Abstracts, ACS, Div. Biol. Chem. April 1956, p. 31c. (*cf.* Teller, J. D.: Direct, quantitative, colorimetric determination of serum or plasma glucose. Abstracts, ACS, Div. Biol. Chem. September 1956, p. 69c.)

(20) LASZLO, J., STENGLE, J., WIGHT, K., and BURK, D.: Effects of chemotherapeutic agents on metabolism of human acute leukemia cells *in vitro*. Proc. Soc. Exper. Biol. & Med. 97: 127–131, 1958.

(21) BURK, D.: Über die Begründung einer Chemotherapie des Krebses auf der Grundlage einer primären Hemmung der Glucosephosphorylierung (Hexokinase-Reaktion) durch Beeinflussung von Substrat, Coenzym und Enzym. Klin. Wochenschr. 35: 1102–1105, 1957.

(22) WOODS, M. W., LANDY, M., and SHEAR, M. J.: Effects of endotoxic polysaccharides on tumor glycolysis. (Abstract.) Fed. Proc. 18: 355, 1959.

(23) LEPAGE, G. A., and GREENLEES, J. L.: Investigation of diverse systems for cancer chemotherapy screening; incorporation of glycine-2-C^{14} into ascites tumor-cell purines as a biological test system. Cancer Res. Supp. 3: 102–105, 1955.

(*24*) HUNTER, J., and WOODS, M.: Effects of 5-fluorouracil and prednisolone on the growth of S91 melanoma. (Abstract.) Proc. Am. Assoc. Cancer Res. 3: 29, 1959.

(*25*) WOODS, M., BURK, D., and HUNTER, J.: Methotrexate, 5-fluorouracil, and prednisolone as inhibitors of tumor glycolysis. (Abstract.) Proc. Am. Assoc. Cancer Res. 3: 74, 1959.

(*26*) GRAFF, S., ENGLEMAN, M., and GRAFF, A. M.: Experiments on the nucleic acids. Acta Unio internat. contra cancrum 7: 463–465, 1951.

(*27*) MANDEL, H. G.: Incorporation of 8-azaguanine and growth inhibition in *Bacillus cereus*. J. Biol. Chem. 225: 137–150, 1957.

(*28*) SARTORELLI, A. C., LEPAGE, G. A., and MOORE, E. C.: Metabolic effects of 6-thioguanine. I. Studies on thioguanine-resistant and -sensitive Ehrlich ascites cells. Cancer Res. 18: 1232–1239, 1958.

(*29*) FINKELSTEIN, M., WINTERS, W. D., THOMAS, P. A., DAVIDSON, C., and SMITH, P. K.: The effect of 8-azaguanine on tissue metabolism in mice bearing sarcoma 37. Cancer Res. 11: 807–810, 1951.

(*30*) HOBBY, G., BURK, D., and LASZLO, J.: Unpublished data.

(*31*) HOCHSTEIN, P.: Hormonal regulation of subcellular glycolysis in the S91 mouse melanoma. *In* Pigment Cell Biology. New York, Academic Press, Inc., 1959, pp. 445–453.

(*32*) WOODS, M., HUNTER, J., and BURK, D.: Regulation of glucose utilization in tumors by a stress-modified insulin : anti-insulin system. J. Nat. Cancer Inst. 16: 351–404, 1955.

(*33*) HUNTER, J. C.: Differential effects of temperature on the growth of certain transplanted tumors in strain DBA mice. J. Nat. Cancer Inst. 16: 405–414, 1955.

(*34*) WOODS, M. W., and HUNTER, J. C.: Metabolic and growth responses of mouse melanomas, Krebs-2 carcinoma, and brain relative to stress, and insulin : anti-insulin hormones. *In* Pigment Cell Biology. New York, Academic Press, Inc., 1959, pp. 455–488.

(*35*) BURK, D., WIGHT, K., and WOODS, M.: Insulin : anti-insulin regulation of metabolism in selective mechanisms of cancer chemotherapy, among a spectrum of malignancies. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 285, 1958.

A Chemical Basis for Tumor-Host Relations[1]

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SUMMARY

Recent work on toxohormone, with emphasis on studies performed in Japan, is reviewed. These studies lend additional support to the concept that systemic effects in the tumor-bearing host involve the alteration of the enzyme level of normal tissue toward the direction of that of tumor tissue (Second Greenstein Law). Alterations of properties in the tumor-bearing host other than enzyme activities, such as involution of the thymus and decrease in liver ferritin, plasma iron, and diphosphopyridine-nucleotide synthesis, have been produced, in recent investigations, by the administration of toxohormone. These studies provide a chemical basis for further study of the effects of tumors on the host.—J. Nat. Cancer Inst. 24: 77–86, 1960.

GREENSTEIN LAW AND SECOND GREENSTEIN LAW IN BIOCHEMISTRY OF CANCER

TWO IMPORTANT generalizations were made by Jesse P. Greenstein (*1*) on the biochemical aspect of neoplastic disease: one concerned cancer tissue itself, and the other the tissues of the cancer-bearing host. Extensive data were collected, showing that cancer tissues, regardless of the normal tissue of origin, tend to converge to a common enzymatic pattern, and this generalization was referred to by Weinhouse (*2*) and Potter (*3*) as the Greenstein Hypothesis, and as the Greenstein Law by Nakahara (*4*). Some objection was raised to this hypothesis or law on the grounds that cancer cells retain special functions and susceptibilities of their tissues of origin to varying degrees and, especially, that there are considerable differences in response among cancers to chemotherapeutic agents. As pointed out by Fukuoka (*5*), however, these differences do not contradict the Greenstein Law. It seems ". . . perfectly reasonable that different tumors may be different in various minor particulars and yet agree as to some important biochemical characters. It may be like all the species belonging to one genus agree as to the generic characters and still have their own specific characters."

[1] Received for publication July 8, 1959.

Greenstein's generalization concerning the tissues of tumor-bearing animals, also based on massive evidence, is that the biochemical characters of the tissues gradually change toward those of the tumor tissue. He stated, "Tumors, in a general sense, apparently present in themselves not only a rather uniform set of chemical components but also produce a more or less uniform effect on the hosts which contain them," and especially pointed out that the changes in the enzymatic pattern of the liver of the tumor-bearing animals are in the direction that the liver would take if it were transformed into a hepatoma. This generalization of "systemic 'cancerousness' " of the tissues of tumor-bearing animals Nakahara (*4*) proposed to call the Second Greenstein Law. This generalization holds also in the water and lipide levels of the trachea-larynx, thymus, lymph nodes, salivary glands, and diaphragm, according to Boyd and associates (*6*), who found that in these levels the tissues of the tumor-bearing animals gradually acquire, to some varying extent, that sameness of composition characterizing the tumor. They suggested that the tumor produced a hydrolipotropic factor whose effect on the tissues of the host is to make their water and lipide concentrations more like those in the tumor itself.

It is this Second Greenstein Law that constitutes a basic principle in tumor-host relations, as I shall attempt to show in this informal, synthesizing paper, based for the most part on work already published, especially from Japanese laboratories.

SYSTEMIC EFFECTS OF TUMORS AND THE TOXOHORMONE CONCEPT

The systemic effects of tumors, to quote from Greenstein (*1*), ". . . may be produced either by a liberation into the blood stream of material elaborated by the tumor or by an abstraction by the tumor of some component or components in the blood stream essential for the maintenance and proper function of the distant tissue." The first of these mechanisms refers to the toxohormone concept, while the second mechanism may be visualized as nutritional inadequacy resulting from the demands of the tumor at the expense of the host.

The relative importance of the two mechanisms in bringing about the deterioration of the host organism is difficult to evaluate exactly, and it is possible that both are operative at the same time. It must be pointed out, however, that nothing specific has been discovered so far regarding the nutritional requirements of the growing tumor tissue. It is common knowledge that tumor-bearing animals continue to consume an adequate amount of food until the tumor reaches a certain size, when they gradually cease to eat and then die in a few days. These circumstances may well suggest some toxic effects; yet, previous investigators have chosen to stress the idea that the deterioration of the host may be due to nutritional insufficiency—largely ignoring the toxic effect. Is cancer death simply a death from starvation? Were it so, it would seem

possible to prevent the death from cancer by parenterally supplying the required food.

It has long been suspected that cancer tissue may produce some toxic substance, but the demonstration of such a substance had to wait until an adequate criterion for its assay was suggested by the work of Greenstein and associates (*7–10*) on the depression of the liver-catalase activity in tumor-bearing animals.

They demonstrated that the liver-catalase decrease was progressive with and proportional to the growth rate of the tumor and that the extirpation of the tumor caused the rapid return to normal of the liver catalase, which was again lowered with re-implantation of the tumor. They also found that the liver-catalase activity of pregnant mice and of the mice bearing large, progressively growing embryonic tissue grafts was normal, which showed that the effect on liver catalase was not due to any growing tissue within the body but was specifically related to the malignant nature of the growing tissue. All these facts pointed to the production by tumor tissue of a substance lowering the liver-catalase level.

It is fair to state that the catalase-depressing tumor substance in question was not demonstrated until 1948, when Nakahara and Fukuoka (*11, 12*) first succeeded in separating it from a variety of human malignant tumors in a crude yet sufficiently concentrated state to be biologically assayable. The designation of toxohormone was proposed for the substance, with the term cancer toxin purposely avoided. It was conceived of as the pathological counterpart of hormones, its presence producing a biochemical lesion in the target organ and its deletion restoring the normal state. "The choice of term toxohormone . . . is a happy as well as pictorial one. It is a circulating factor, and the term expresses the thought very well indeed" [Greenstein (*13*)].

Briefly described, toxohormone is produced by living malignant tumor cells of all types and is in all probability a polypeptide. It is released into the circulation and produces certain biochemical lesions, *e.g.*, reduction of liver catalase, in the target organ, *e.g.*, liver, of the host organism. It is not a decomposition product of degenerating tumor tissue. Toxohormone can be prepared as active concentrates from tumor tissue by extracting it with water and then precipitating it with alcohol from the extract after removing heat-coagulable matter. It may be further purified by reprecipitation with one of the conventional protein precipitants, or by fractionation, with cellulose column chromatography and/or benzoic acid adsorption techniques. The activity of toxohormone is assayable by the marked decrease of the liver catalase, which it produces 24 hours after injection into normal mice, but toxohormone does not affect the catalase activity *in vitro*.

This last point is important in clearing up the misunderstanding that continues to persist, even in recent literature. The toxohormone effect is manifested by the suppression of the catalase synthesis *in vivo*, and not through the direct inactivation of catalase molecules. Many agents are now known that lower the activity of liver catalase when they are injected

into normal animals: sulfhydryl-containing tissue kochsaft (*14*) or homogenates (*15, 16*), cystine derivatives (*17, 18*), 3-amino-1,2,4-triazole (*19*), etc., but these agents inhibit catalase activity *in vitro* also, and they should not be confused with tumor toxohormone, which has no effect on catalase *in vitro*. That the depression of liver-catalase activity *in vivo* produced by these nonspecific agents must be clearly distinguished from the toxohormone effect has been emphasized by the work of Endo *et al.* (*20*) on tissue kochsaft, and of Sugimura (*21*) on 3-amino-1,2,4-triazole.

The situation has been confused only by some of those investigators who have used whole kochsaft or homogenate of tissues, but never by those who have experimented with isolated toxohormone concentrates.

For a recent review of the literature on toxohormone the reader is referred to Nakahara and Fukuoka (*22*).

TOXOHORMONE EFFECT ON IRON METABOLISM

To evaluate toxohormone in tumor-host relations it is necessary to establish the type of biological effects that can be produced in normal animals by the injection of isolated toxohormone. The well-documented effects are enumerated, with typical examples of experimental data quoted from various published papers, as follows:

Depression of Liver-Catalase Level

Mice	Average liver-catalase activity (O_2 cc.)
Normal (40)	8. 0
Injected with toxohormone (36)	4. 3
Injected with "toxohormone fraction" from normal tissues (24)	7. 2

The data quoted are based on injections of alcohol precipitate originally described by Nakahara and Fukuoka (*11*). The liver-catalase level is expressed in cc. of O_2 by the gas-volumetric method, determined at 20 hours after a single injection of 50 to 100 mg. of the fractions.

Increase of Liver-Protoporphyrin

Rats	Average protoporphyrin concentration in liver ($\mu g.$/100 $gm.$)
Normal (13)	16. 0
Tumor-bearing (9)	30. 0
Injected with toxohormone (9)	22. 1

Ono, Umeda, and Sugimura (*23*), in my laboratory, demonstrated a distinct increase of protoporphyrin in the liver of tumor-bearing and of toxohormone-injected rats, by the use of Umeda transplantable fibrosarcoma

and the toxohormone fraction prepared from the same tumor, according to the method of Nakahara and Fukuoka. In the experiment yielding the data quoted, toxohormone was injected in amounts (300–400 mg.) sufficient to produce a marked liver-catalase depression. The method of Schwartz *et al.* was used for the analysis of porphyrin, the protoporphyrin concentration in the extracts being determined at 411.5 mμ by means of the Beckman spectrophotometer.

Decrease of Liver Ferritin

	Average non-hemin iron in the liver (γ/*gm. wet wt.*)			
Mice	Hemosiderin	Ferritin	Nucleoprotein-bound	Free
Normal (5)	44	90	9	1
Injected with toxohormone (5)	39	50	12	1

Paralleling the fact that in tumor-bearing animals there is a lowering in the amount of storage iron in the liver, Hoshizima (*24*) observed a marked decrease in the liver ferritin after an injection of toxohormone into normal mice. Non-hemin iron other than ferritin was not affected. The sample of toxohormone used was the alcohol precipitate obtained from surgically removed human gastric-cancer tissue, and the method for the determination of non-hemin iron was that of Yoneyama and Konno.

Decrease of Plasma Iron

Rats	Average plasma iron (*μg. percent*)
Normal (10)	294
Injected with toxohormone	
1 mg., 8 hours before (5)	56
100 mg., 10 hours before (10)	29
100 mg., 24 hours before (8)	62

In a recent publication, Kampschmidt, Adams, and McCoy (*25*) presented evidence of extraordinary decrease in plasma iron after injections of toxohormone (alcohol precipitate) from Walker carcinosarcoma 256. No such decrease in plasma iron was induced by a similar preparation obtained from normal rat tissues. The plasma iron decrease appeared in rats 8 to 24 hours after a single injection of 0.5 to 100 mg. of toxohormone, the plasma iron reverting back to the normal level in 48 hours. The method used for the determination of plasma iron was that of Schade, Oyama, Reinhart, and Miller.

Although it has been known that animals with tumors show low plasma iron, the extremely low value observed precipitously, after a single injection of toxohormone, was unexpected. This remarkable finding has already been confirmed by Ono and Ohashi (*26*), in my laboratory, and, as Kampschmidt *et al.* suggested, the measurement of plasma iron, now found to be more sensitive than is the liver catalase, may prove to be highly valuable as a bioassay system for toxohormone.

The outstanding systemic changes so far established in tumor-bearing animals are specifically related to the disturbance of iron metabolism, and it is significant that these changes are reproducible in normal animals by the injection of toxohormone isolated from tumor tissues. The depression of liver-catalase synthesis, with corresponding increase of liver protoporphyrin, and the decrease of liver ferritin and of plasma iron seem to be closely interrelated phenomena, and as such they speak strongly for the implication of toxohormone in the disturbed iron metabolism in tumor-bearing animals.

The exact relations of these several changes to each other in the actual process of toxohormone effect as it takes place *in vivo* are not precisely definable at the present time. Catalase, as well as hemoglobin and cytochromes, is a hemoprotein, composed of iron-porphyrin compound and protein, and the remarkable increase in the hepatic protoporphyrin, concomitant with the decrease of liver catalase, clearly indicates that there is a definite suppression of the utilization of porphyrin for the synthesis of catalase. It is known that porphyrins do not bind ferrous or ferric ions spontaneously, but that iron is inserted into the porphyrin by an enzymatic process involving a preparatory enzyme-porphyrin combination, or is bound to a porphyrin precursor with subsequent macrocyclization about the metal iron. The toxohormone effect, which apparently leaves much protoporphyrin unutilized for catalase synthesis, suggests that it may involve the enzymatic process of iron insertion into the porphyrin.

Ferritin does not contain porphyrin: Its iron component is combined to the protein moiety (apoferritin) and is assumed to be important in iron metabolism. Hoshizama (*24*), finding that the depression of catalase activity in the same liver was paralleled by the reduction of ferritin, suggested that the first response of the iron metabolism in the liver of tumor-bearing host may occur in the ferritin iron, which may be in a closely equilibriated state with liver catalase.

The transitory but precipitous nature of the effect of toxohormone injection on plasma iron, demonstrated by Kampschmidt *et al.*, is in itself a subject worthy of further study, but there is little doubt that it is a part of the whole process of iron metabolism as affected by toxohormone.

If the fundamental concept we have developed is true, that toxohormone acts through its ability of interfering with the utilization of iron, inference is justified that all the iron-containing biochemical systems may possibly be affected by toxohormone. Hemoglobin, cytochromes, and cytochrome oxidase may all come under this category, and, indeed, there is experimental evidence that the hemoglobin level of normal mice is to some extent lowered by injections of toxohormone (*27*). There seem to be differences among these iron-containing active substances in the susceptibility to toxohormone action. The fact that not all the tumor effects have been adequately reproduced by toxohormone injections may simply mean that some of these effects require long-continued action for their manifestations.

Begg (*28*), commenting on the apparent emphasis on catalase by

investigators in this area, raised the question: "Why catalase?" According to him ". . . the reason . . . appears to rest on the ready availability of something that can be measured," and ". . . the emphasis has been on the production of the effect, *not on the meaning of the low enzyme concentration to the host.*" [Italics are mine.] It seems pertinent to add that the fact that liver catalase is most susceptible to toxohormone action does not make it less a part of the entire enzyme system having iron as the prosthetic group, and that the possible consequences of depressed activity of these enzymes are perhaps too obvious to be pointed out. It is indeed from this consideration, rather than mere pragmatism, that the liver-catalase activity has attracted special attention in the study of tumor-host relations. The marked lowering of the synthesis of catalase cannot be expected to occur without concurrently involving changes in general iron metabolism, and further studies along this line may be expected to yield a key to the understanding of abnormal iron metabolism and all that it involves in tumor-bearing organisms.

TOXOHORMONE EFFECTS APPARENTLY NOT CONNECTED WITH IRON METABOLISM

In tumor-bearing animals, there are other systemic changes which cannot be considered in direct connection with the disturbance of iron metabolism. These include, besides other changes that are either slight or are of somewhat irregular occurrence, two types of well-marked changes constantly associated with tumors: (1) depression of the diphosphopyridine nucleotide (DPN) synthesis in the liver, and (2) the involution of the thymus. These two well-documented changes in tumor-bearing animals can be readily reproduced in normal animals by injections of toxohormone.

Depression of Diphosphopyridine Nucleotide Synthesis in the Liver

Mice	Average amount of DPN in liver, 8 hours after nicotinamide injection (*mg./gm.*)
Normal (20)	2. 23
Pregnant (5)	2. 01
Tumor-bearing, Sarcoma 180 (5)	1. 52
Tumor-bearing, N-F sarcoma (5)	1. 66
Toxohormone-injected, 70 mg. (5)	1. 25

Waravdekar (*29*) first reported that the DPN-synthesizing activity of the liver was lower in tumor-bearing animals than in normal controls, and suggested the existence of humoral factor responsible for this change. Ono and Tomaru (*30*), in my laboratory, recently demonstrated that the increase of DPN in the liver, induced by nicotinamide administration, was markedly inhibited 24 hours after an injection of toxohormone (alcohol precipitate fraction) in doses sufficient to depress the liver-

catalase activity. The degree of the inhibition was more than equal to that brought about by the presence in the host of growing tumors.

A similar inhibiting effect, with only slight variations depending on the material injected, on the DPN-synthesizing activity of the liver was also found by these authors, with other toxohormone preparations. In these studies the authors used the method of Jedkin and Weinhouse, the amount of DPN being measured by the absorption at 340 mμ after the addition of alcohol dehydrogenase and ethanol in 1/30 M pyrophosphate buffer at *p*H 9.0. For inducing DPN synthesis in the liver, nicotinamide was injected intraperitoneally into mice in doses of 50 mg. per kg. of body weight, 8 hours before making the DPN determination.

Ono and Tomaru failed to confirm Waravdekar's original observation that the homogenate of tumor and the liver of tumor-bearing animals inhibit the DPN-synthesizing enzyme *in vitro*. Toxohormone preparations also proved inactive in tests *in vitro*. Obviously, the tumor and toxohormone effect on the synthesis of liver DPN is limited to the inhibition of the synthesis *in vivo*, which shows a remarkable parallelism to the effect on the liver-catalase activity.

Involution of the Thymus

Mice	Average thymus weight (*mg./100 gm. body weight*)
Normal (25)	130. 2
Tumor-bearing (28)	45. 7
Toxohormone-injected (10)	35. 1

The marked involution of the thymus is a well-known phenomenon in tumor-bearing mice and rats, and it can be easily produced in normal mice or rats by a single injection of toxohormone (*31*). The data tabulated were obtained 24 hours to 3 days after the injection of toxohormone isolated from Nakahara-Fukuoka strain of transplantable mouse sarcoma in doses sufficient to depress the liver-catalase activity.

An important question arising from the survey of toxohormone effects is that of the possible plurality of toxohormone. Although all the toxohormone concentrates so far tested were found to be capable of suppressing DPN synthesis and of inducing the involution of thymus, in addition to interfering with the utilization of iron at the same time, it is not readily acceptable that these diverse effects are attributable to a single chemical substance. The possibility cannot be excluded, however, that a single toxohormone can act in such a way as to inhibit several synthetic processes—some of them more directly and others more indirectly. Only further progress in the purification of toxohormone can settle this moot question.

CONCLUSION

The elaboration and release of toxohormone is an attribute common to all the malignant tumors, so far as our knowledge extends, and it is

through this property that all the malignant tumors agree in producing certain common systemic effects on tumor-bearing hosts. As postulated by the Second Greenstein Law, the systemic effects concerned here involve the alteration of the enzymic level of normal tissue (liver) toward the direction of that of tumor tissue, and in toxohormone we find a chemical basis for this important aspect of tumor-host relations.

Although the study arose from observations on the enzyme catalase in the liver of tumor-bearing mice and rats, so much additional evidence has been accumulated that the whole subject now appears to afford a clue to one of the fundamental phenomena in neoplastic disease. Cancer as a disease is a case of "autoparasitism," and just as parasite-host interactions form the basis of the pathology of infectious diseases, ". . . the host-tumor relationship is the key to the cancer problem" (*1*). If toxohormone is indeed a cancer toxin in the true sense of the word, further studies of toxohormone and its effects may be expected to contribute to the understanding of the mechanism which leads to cachexia and ultimate death of tumor-bearing organisms.

REFERENCES

(*1*) GREENSTEIN, J. P.: Biochemistry of Cancer, 2d ed. New York, Academic Press, Inc., 1954.

(*2*) WEINHOUSE, S.: Discussion of Doctor Greenstein's paper. Cancer Res. 16: 654–657, 1956.

(*3*) POTTER, V. R.: Biochemical uniformity and heterogeneity in cancer tissue. (Further discussion.) Cancer Res. 16: 658–667, 1956.

(*4*) NAKAHARA, W.: Proceedings of the conference on biochemistry of cancer. Gann 48: 110–111, 1957.

(*5*) FUKUOKA, F.: Proceedings of the conference on biochemistry of cancer. Gann 48: 111, 1957.

(*6*) BOYD, E. M., KELLY, E. M., MURDOCH, M. E., and BOYD, C. E.: Lipid and water levels in 5 organs of albino rats bearing Walker carcinosarcoma 256. Cancer Res. 16: 535–543, 1956.

(*7*) GREENSTEIN, J. P., JENRETTE, W. V., and WHITE, J.: The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. J. Nat. Cancer Inst. 2: 283–291, 1941.

(*8*) GREENSTEIN, J. P., and ANDERVONT, H. B.: The liver catalase activity of tumor-bearing mice and the effect of spontaneous regression and of removal of certain tumors. J. Nat. Cancer Inst. 2: 345–355, 1942.

(*9*) GREENSTEIN, J. P.: Further studies of the liver catalase activity of tumor-bearing animals. J. Nat. Cancer Inst. 3: 397–404, 1943.

(*10*) GREENSTEIN, J. P., and ANDERVONT, H. B.: Note on the liver catalase activity of pregnant mice and of mice bearing growing embryonic implants. J. Nat. Cancer Inst. 4: 283–284, 1943.

(*11*) NAKAHARA, W., and FUKUOKA, F.: A toxic cancer tissue constituent as evidenced by its effect on liver catalase activity. Jap. M. J. 1: 271–277, 1948.

(*12*) ———: Toxohormone: a characteristic toxic substance produced by cancer tissue. Gann 40: 45–69, 1949.

(*13*) GREENSTEIN, J. P.: Proceedings of the conference on biochemistry of cancer. Gann 48: 111–112, 1957.

(*14*) HARGREAVES, A. B., and DEUTSCH, H. F.: The *in vitro* inhibition of catalase by a tumor factor. Cancer Res. 12: 720–726, 1952.

(*15*) Day, E. D., Gabrielson, F. C., and Lipkind, J. B.: Depressions in the activity of liver catalase in mice injected with homogenates of normal mouse spleen. J. Nat. Cancer Inst. 15: 239–252, 1954.

(*16*) Riley, E. E.: Depression of liver catalase by various agents. Cancer Res. 19: 285–291, 1959.

(*17*) Seabra, A., and Deutsch, H. F.: Studies on the catalase inhibition as related to tumors. J. Biol. Chem. 214: 447–454, 1955.

(*18*) Hirai, H., and Deutsch, H. F.: The effect of some cystine derivatives on catalase activity. Cancer Res. 18: 283–288, 1958.

(*19*) Heim, W. G., Appleman, D., and Pyfrom, H. T.: Production of catalase changes in animals with 3-amino-1, 2, 4-triazole. Science 122: 693–694, 1955.

(*20*) Endo, H., Sugimura, T., Ono, T., and Konno, K.: Catalase depressing tissue factors: toxohormone and kochsaft factor. Gann 46: 51–56, 1955.

(*21*) Sugimura, T.: The mechanism of liver catalase depression by 3-amino-1,2,4-triazole. Gann 47: 159–170, 1956.

(*22*) Nakahara, W., and Fukuoka, F.: The newer concept of cancer toxin. Advances Cancer Res. 5: 157–177, 1958.

(*23*) Ono, T., Umeda, M., and Sugimura, T.: Porphyrin metabolism in tumor-bearing animals. Free porphyrin in liver, Harderian gland and urine and effect thereon of toxohormone. Gann 47: 171–180, 1956.

(*24*) Hoshizima, H.: Studies on the mechanism of toxohormone function. Gann 48: 239–242, 1957.

(*25*) Kampschmidt, R. F., Adams, M. E., and McCoy, T. A.: Some systemic effects of toxohormone. Cancer Res. 19: 236–239, 1959.

(*26*) Ono, T. and Ohashi, M.: Unpublished observation.

(*27*) Nakahara, W., and Fukuoka, F.: Unpublished observation.

(*28*) Begg, R. W.: Tumor-host relations. Advances Cancer Res. 5: 1–54, 1958.

(*29*) Waravdekar, V. S., and Powers, O. H.: Reduction of synthesis of diphosphopyridine nucleotide in tissues from mice bearing transplanted tumors. J. Nat. Cancer Inst. 18: 145–152, 1957.

(*30*) Ono, T., and Tomaru, T.: The toxic tumor factor depressing DPN synthesis in tumor-bearing animals. Gann 50: 37–45, 1959.

(*31*) Fukuoka, F., and Nakahara, W.: Toxohormone and thymus involution in tumor-bearing animals. A fourth study on toxohormone, a characteristic toxic substance produced by cancer tissue. Gann 43: 55–62, 1952.

Studies on the Anemia of Tumor-Bearing Animals. IV. Distribution of Radioiron After the Extravascular Injection of Labeled Erythrocytes [1,2]

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SUMMARY

Previous studies (*1–3*) have shown that certain rat tumors contain large amounts of iron which arise primarily from hemoglobin iron in hemorrhagic areas of the tumor. The present studies attempt to determine to what degree the host is able to reutilize this iron for red-cell synthesis. Because of the complexity of the problem in tumors, initial studies were made on the fate of iron from extravascularly injected cells in normal rats. When intact erythrocytes which had been labeled with Fe^{59} were injected into the gastrocnemius muscle of normal rats, the radioiron left the site of injection very slowly, at a rate of 1 percent per day. After 60 days only 18 percent had been reutilized for the synthesis of new red cells. The rate at which radioiron from labeled cells left the injection site in the leg was only slightly increased in tumor-bearing animals or in animals which had been made anemic by repeated hemorrhage. In contrast, when erythrocytes containing Fe^{59}-labeled hemoglobin were lysed and injected into the gastrocnemius muscle of normal rats, 55 percent of the radioiron had left the injection site within 24 hours and 35 percent was recovered in the liver. In later experiments, a slow rate was noted when intact cells labeled with Fe^{59} were injected into the tumor tissue of tumor-bearing animals and into the subcutaneous tissue of normal animals. The slow reutilization of iron from injected blood, in these experiments, suggests that the iron from extravasated blood may also be only slowly reutilized in normal and tumor-bearing rats.—J. Nat. Cancer Inst. 24: 87–96, 1960.

THE PRESENT paper is part of a program carried out in this laboratory on the iron metabolism of normal and tumor-bearing rats. Dr. Greenstein's extensive studies on the relationship between the growth of tumors and the reduction in activity of the iron-containing enzyme, catalase, in the liver of the host (*4*), stimulated our initial interest in this field; his continued interest and support were important in the development of the

[1] Received for publication August 3, 1959.

[2] The results of parts of this investigation were presented at the Seventh International Cancer Congress, London, England, July 1958, and the 50th Annual Meeting of the American Association for Cancer Research, Atlantic City, N.J., April 1959.

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[4] The authors wish to express their appreciation to Mary E. Washko and Bessie M. Watkins for their skillful assistance.

[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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experimental program. It is appropriate that a paper of this series to which he contributed so much should be published in a memorial issue in his name. Previous studies (*1–3*) in this series have shown that the anemia associated with any one of a number of animal tumors could be explained primarily by a loss of red cells into the tumor, and have indicated that in various tumors different vascular defects accounted for the loss of erythrocytes. Those tumors associated with the most rapid onset of anemia, such as the Lymphosarcoma[1]R2788 of the rat, were seen to have a narrow band of hemorrhage between the tumor and the connective tissue of the capsule. In the connective tissue adjacent to the hemorrhage, many macrophages were observed, containing high concentrations of iron in the form of hemosiderin. Other tumors that were associated with a more gradual onset of anemia showed evidence of hemorrhage into the necrotic areas, as seen in Hepatoma 3683 of the rat, or the trapping of blood in thrombosed sinusoids, as seen in the spontaneous mammary tumor of C3H mice. In each case, irrespective of the histological findings, a major fraction of the iron lost from the red cells during the development of the anemia could be recovered in the tumor.

As a result of these studies, a question has arisen as to how long iron from extravasated blood remains in the area of the tumor. Is it sequestered in the tumor for a long time, or is it quite rapidly returned to the active iron pool of the body? Since the loss of red cells into the area of the tumor is a progressive process which increases with the growth of the neoplasm, it is difficult to obtain any precise estimate of the rate at which the erythrocyte iron, which is deposited in the tumor at any given time, again becomes available for erythrocyte synthesis. Because of the complexity of the problem in tumors, a simpler system was required in which the number of variables could be more readily controlled. Therefore studies were first carried out to determine the fate of iron from intact and lysed erythrocytes when injected into the gastrocnemius muscle of normal rats. Subsequently, the fate of iron from blood injected subcutaneously and directly into tumor tissue was investigated. The results of these studies are presented herein.

MATERIALS AND METHODS

The labeled blood used in earlier experiments was prepared by daily intraperitoneal injection, for 10 days, of 21 μc. of Fe^{59}, as ferric ammonium citrate, into female Fischer rats weighing about 180 gm. In later experiments uniformly labeled blood was prepared in male M520 $\times$ (A $\times$ C/9935) hybrid rats, by the injection of 10 μc. of Fe^{59} 3 times a week for 3 months, starting with weanling rats weighing approximately 50 gm.

Twenty-four hours after the last injection into the donor rats, blood was withdrawn from the bifurcation of the aorta into a heparinized syringe, and aliquots containing 14 mg. of hemoglobin and approximately 2 μc. of Fe^{59} in a volume of 0.1 ml. were injected into the gastrocnemius

muscle of the right leg of recipient rats of the corresponding strain. At appropriate intervals, groups of 2 or 3 animals were anesthetized and a sample of blood was obtained from each, on which the radioiron concentration was determined. These animals were killed immediately after the sample was taken, and the livers and spleens were removed. The hind legs were carefully dissected from the body, and the remaining carcasses were minced. The tissues were subsequently digested by heating in 30 percent KOH, and aliquots of the digests were weighed into vials in which the concentration of Fe^{59} was measured with a scintillation counter.[6] Because of the difficulty of injecting consistently 0.1 ml. into these animals, all results are expressed as percent of the total recovered activity. Actual recoveries ranged from 83 to 104 percent of the injected Fe^{59}. The total amount of Fe^{59} in the blood was estimated by assuming a blood volume of 5 ml. per 100-gm. body weight.

In a second series of experiments, cells containing hemoglobin labeled with Fe^{59} were lysed and injected into the gastrocnemius muscle of normal rats. At intervals after the injection, 2 or 3 animals were killed, and the distribution of Fe^{59} was determined as described. In earlier experiments, red cells labeled with Fe^{59}, over a 10-day period, were lysed by dialysing blood against distilled water for 1 hour at room temperature. The solutions were then dialyzed against 0.9 percent NaCl to restore the isotonic saline concentration. The blood was then centrifuged at 10,000 $\times$ g to remove any insoluble stroma or crystalline hemoglobin and injected, in doses of 0.1 ml., into the gastrocnemius muscle of 36 Fischer rats. In later experiments carried out in M520 $\times$ (A $\times$ C) hybrids, uniformly labeled oxalated blood was centrifuged and the plasma was removed. The packed cells were taken to volume with distilled water and were dialyzed against distilled water for 2 hours. The lysed solution was redialysed for 2 hours against 0.15 M acetate buffer *p*H 4.7 to minimize the crystallization of hemoglobin, centrifuged for 10 minutes at 10,000 $\times$ g, and recombined with the original plasma. The reconstituted lysed blood was made 0.01 M as to $CaCl_2$ and 0.1-ml. aliquots containing 6 mg. Hb were immediately injected into the gastrocnemius muscle of 24 rats.

In certain experiments the radioiron in the tissues was separated into a dialyzable and nondialyzable fraction by dialysis of the tissues for 48 hours against 3 changes of 0.1 M sodium hydrosulfite ($Na_2S_2O_4$). Separate experiments had previously shown that under these conditions dialysis of blood labeled with Fe^{59} led to a loss of only 1.5 percent of the radioiron, but that labeled ferritin could be dialyzed free from radioiron. Thus it was possible by this simple dialysis procedure to separate the radioiron remaining in hemoglobin from that which had been converted to heme or nonchelated protein complexes.

Experiments were also carried out in which the distribution of Fe^{59} was determined at intervals after the intramuscular injection of labeled cells into rats that had been repetitively bled. Blood was obtained from

[6] Model DS-3 Nuclear Instrument and Chemical Corporation, Chicago, Ill.

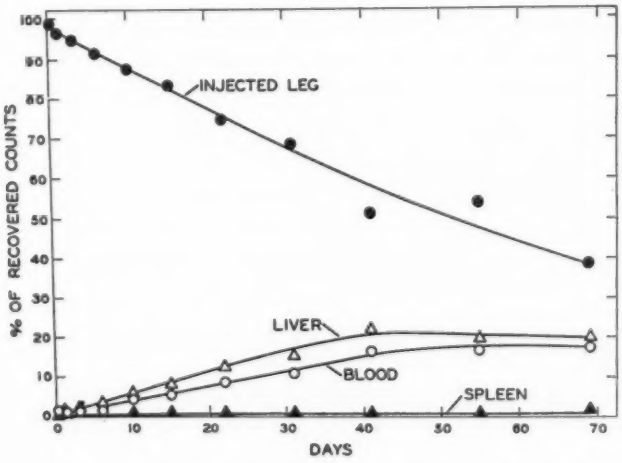
the tail of rats anesthetized with sodium pentobarbital, and collected in 0.9 percent NaCl containing one-half percent heparin.

In other experiments, the Fe^{59} distribution was studied after the injection of labeled blood into the gastrocnemius muscle of tumor-bearing rats, or after the injection of labeled blood directly into the tumor. The Hepatoma 3683 (*5*) and Lymphosarcoma R2788 (*6*) tumors used in these experiments were carried in the hybrid M520 × (A × C/9935) rats and were transplanted by injecting, with a trocar, 3 small pieces of tumor bilaterally in the lumbar region. Direct injection into the tumor was made through the paravertebral muscles to prevent leakage of blood back through the injection site. All animals were fed *ad libitum* with Bryant mouse pellets (7).

EXPERIMENTS

Distribution of radioiron following the injection of whole cells labeled with Fe^{59} into the gastrocnemius muscle of normal rats.—The results obtained after the injection of whole cells into the gastrocnemius muscle of normal Fischer rats are presented in text-figure 1. The radioiron left the site of injection at a rate of about 1 percent a day. At the end of 30 days, 69 percent of the radioiron remained at the site of injection, 15 percent had entered the liver, and only 11 percent had entered the blood; the remaining carcass had 4.5 percent.

At the end of 69 days, 39 percent of the radioiron remained at the site of injection, and only 18 percent was recovered in the circulating blood. The radioiron in the liver amounted to 20 percent, in the spleen 2 percent, and in the remaining carcass 21 percent.

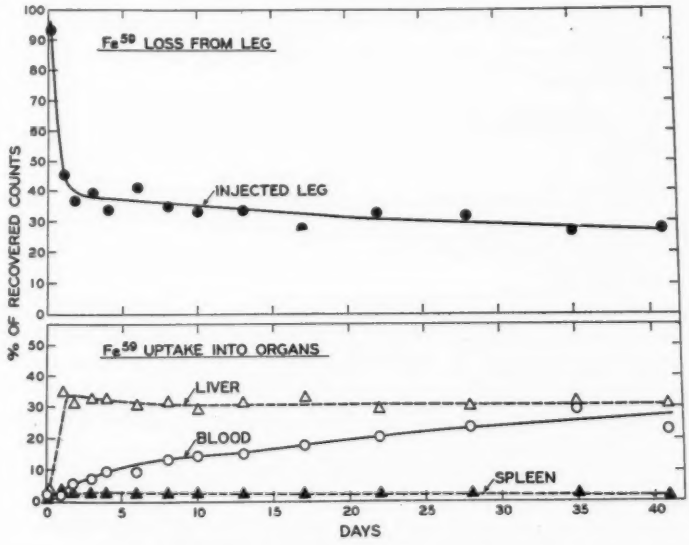


TEXT-FIGURE 1.—Distribution of radioiron after the injection of intact red cells labeled with Fe^{59} into the gastrocnemius muscle of normal rats.

Other studies (*8*) in this laboratory have shown that the young red cell is much more resistant to osmotic hemolysis than the more mature red cell. To rule out the possibility that the slow rate of iron return might arise from the fact that only relatively immature cells were labeled, the experiments described were repeated with uniformly labeled cells in male M520 × (A × C) hybrid rats. Although, in this instance, the spread of experimental points was somewhat greater, the slope of the line describing the loss of radioiron and the distribution of the radioiron was similar.

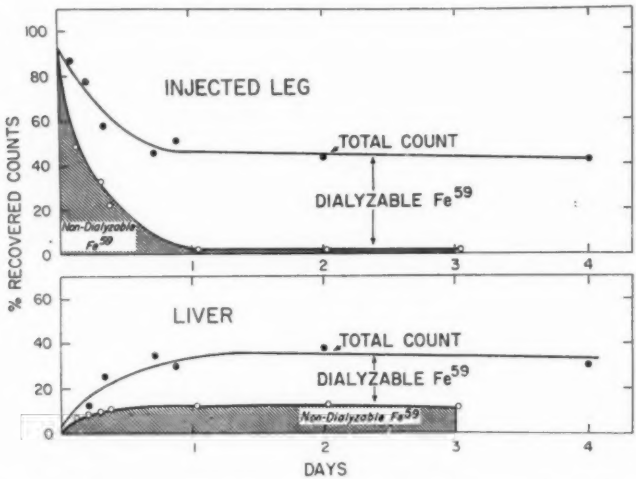
Distribution of radioiron after the injection of lysed cells labeled with Fe^{59} into the gastrocnemius muscle of normal animals.—The results obtained after the injection of lysed cells into the gastrocnemius muscle of normal Fischer rats are presented in text-figure 2. In contrast to the slow continuous loss of radioiron from the site of injection in the experiment with whole cells, 55 percent of the radioiron left the site of injection within 24 hours after the injection of lysed cells. At this short interval over 35 percent of the radioiron was recovered in the liver, 2 percent was in the blood, and only 2.7 percent was in the spleen. After 24 hours, there was a sharp decrease in the rate at which radioiron left the site of injection. The proportion of the radioiron in the liver and spleen did not vary much during the remaining 41 days of the experiment. At the end of 42 days approximately 25 percent of the radioiron, or about one third of that which had left the site of injection, was recovered in the blood.

The rather marked decrease at 24 hours in the rate at which the iron from lysed cells left the point of injection, in this experiment, was studied further. Because of the ease with which the hemoglobin of the rat can



TEXT-FIGURE 2.—Distribution of radioiron after the injection of hemolyzed cells labeled with Fe^{59} into the gastrocnemius muscle of a normal rat.

be crystallized in normal saline, it was initially postulated that a portion of the injected hemoglobin had crystallized in the tissue. The experiment was repeated with uniformly labeled oxalated blood which had been dialyzed against 0.1 M acetate buffer, *p*H 4.7, to minimize hemoglobin crystallization. In addition, tissue taken at various intervals after the injection of lysed cells were dialyzed against 0.1 M sodium hydrosulfite. The results of these experiments are shown in text-figure 3. The loss of radioiron from the injected leg and uptake by the liver described curves similar to those observed previously. It can be seen that after 24 hours 97 percent of the iron remaining in the injected leg was dialyzed from the tissue with 0.1 M sodium hydrosulfite. As iron cannot be dialyzed from hemoglobin or catalase under these conditions, but can be readily separated from ferritin or hemosiderin, it would seem unlikely that much of the iron remaining in the muscle was in the form of hemoglobin, after 24 hours.



TEXT-FIGURE 3.—Distribution of radioiron on dialysis of tissues against 0.1 M $Na_2S_2O_4$ at various intervals after the injection of hemolyzed cells labeled with Fe^{59} into the gastrocnemius muscle of a normal rat.

At the 1-, 2-, and 3-day period, 13, 12, and 11 percent, respectively, of the total recovered radioactivity was found in the nondialyzable fraction of the liver. This amount of radioiron greatly exceeds the amount of iron which can be accounted for by circulating blood remaining in the liver. These findings suggest either that the iron from the lysed red cells was transported to the liver as hemoglobin where it was more slowly broken down than in the muscle, or that the iron was rapidly incorporated into a chelated-protein complex in the liver.

Distribution of radioiron after the injection of intact erythrocytes labeled with Fe^{59} into the gastrocnemius muscle of bled animals.—To determine whether a loss of red cells similar to that observed in certain tumor-bearing

animals (*1*) had an effect on the rate at which whole cells left the gastrocnemius muscle, 24 hybrid rats with an average weight of 140 gm. received an injection of 0.1 ml. of uniformly labeled cells, and subsequently half the animals were bled 3 ml. or approximately 40 percent of their blood volume. An equal amount of plasma was injected intraperitoneally to prevent shock. At 4-day intervals the bleeding was repeated 5 times. The animals did not recover their previous hemoglobin levels between bleedings, though there was considerable erythropoiesis during this period. Consequently 4 days after the last removal of blood the hemoglobin level was 60 percent of normal. At intervals of 6, 12, 20, and 30 days after the injection of labeled cells, 3 animals of each group were killed, and the distribution of isotope was determined. At the initial point of 6 days, the amount of Fe^{59} remaining at the site of injection was 12 percent less in the bled animals than in the controls. At succeeding intervals this difference remained almost constant and the rate of loss of both groups of animals after the initial interval was similar and agreed with the previous rate found after the intramuscular injection of whole cells into normal animals.

At the end of 30 days, 61 percent of the radioiron remained at the point of injection in the normal animal and 54 percent in the bled animal. The normal animal had 24 percent of the radioiron in the liver, 6 percent in the circulating blood, 0.3 percent in the spleen, and 8 percent in the remaining carcass. The bled animal had 21 percent of the radioiron in the liver, 19 percent in the circulating blood and the blood withdrawn, 0.4 percent in the spleen, and 5 percent in the remaining carcass.

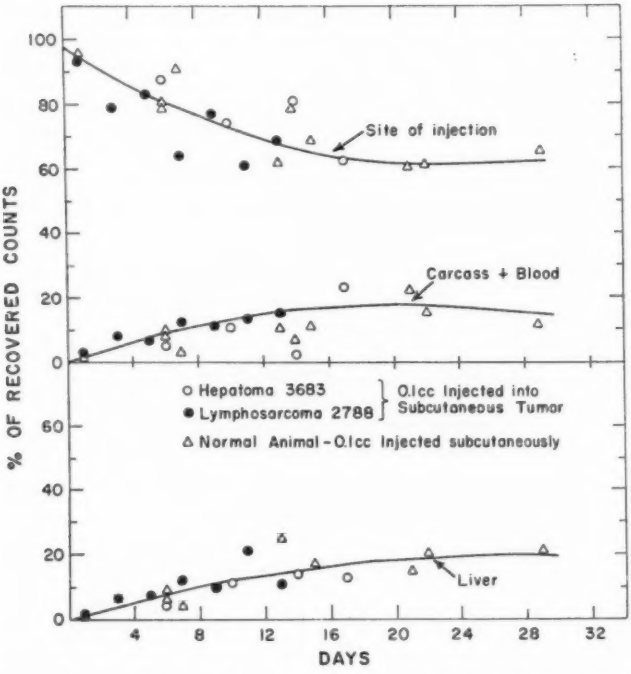
Distribution of radioiron after the injection of erythrocytes labeled with Fe^{59} into the gastrocnemius muscle of tumor-bearing rats.—Rats were injected with labeled red cells and on the same day Lymphosarcoma R2788 was subcutaneously implanted into 10 of them. In this particular group of animals the rate of tumor growth varied markedly. At the end of 22 days, the tumor-bearing animals were grouped on the basis of tumor weight and the distribution of Fe^{59} was compared with that of normal animals (table 1); the percentage of radioiron remaining at the site of injection varied from 65 percent in animals with small tumors to 59 percent in animals with large tumors, as compared to a recovery of 70 percent in

TABLE 1.—Distribution of radioiron 22 days after injection of intact erythrocytes labeled with Fe^{59} into the gastrocnemius muscle of rats subsequently injected with Lymphosarcoma R2788

Average tumor weights (*number of animals*)	— (*3*)	7 (*3*)	21 (*5*)	46 (*2*)
	Percent recovered counts			
Injected leg	70	65	65	59
Blood	2	3	6	10
Tumor	—	1	6	14
Liver	19	16	17	12
Spleen	<1	<1	<1	<1
Carcass	9	14	6	3

the normal rats. Although no large difference was observed in the percentage of radioiron left at the site of injection in tumor-bearing animals, the distribution of the iron in the rest of the body varied markedly with tumor size. The percentage of Fe^{59} in the blood and tumor increased with tumor size, while the percentage in the liver and carcass decreased. This change in the distribution of labeled iron probably reflects the requirement of iron for erythropoiesis, which arises in the host as the loss of red cells into the area of the tumor increases with the growth of the tumor (*1–3*).

Distribution of radioiron after the injection of intact erythrocytes labeled with Fe^{59} into the tumor tissue of tumor-bearing rats.—Hybrid M520 × (A × C) rats were injected with Lymphosarcoma R2788. After 8 days, when the tumor measured 1 or 2 cm. in diameter, 0.1 ml. of labeled red cells was injected into the tumor. At intervals after the injection of labeled cells, groups of 3 animals were killed and the distribution of the iron was determined. On the 14th and 20th day after transplantation, the rats bearing lymphosarcoma had an average tumor weight of 28 and 42 gm., or 18 and 27 percent of the total body weight, respectively. The corresponding hemoglobin concentrations were 4.5 gm. percent and 6.0



TEXT-FIGURE 4.—Distribution of radioiron after the injection of intact erythrocytes labeled with Fe^{59} into the subcutaneous tissue of normal rats and tumor tissue of tumor-bearing rats.

gm. percent compared with an average hemoglobin concentration of 13.3 gm. percent in the control rats.

A similar experiment was carried out on animals bearing Hepatoma 3683, in which labeled cells were injected into the tumor on the 11th day after transplantation. On the 19th and 26th day after transplantation, the rats bearing hepatoma had an average tumor weight of 10.4 and 23 gm. percent, or 6 and 14 percent of the total-body weight, respectively. The corresponding hemoglobin concentrations were 9.2 and 9.5 percent, as compared to an average hemoglobin concentration of 13.5 gm. percent in the control rats.

The results of these experiments are presented in text-figure 4. The iron distribution found at various intervals after the subcutaneous injection of labeled cells in normal animals is plotted for comparison. Within the rather large variability of this experiment, it can be seen that the loss of Fe^{59} from the tumor has a similar rate to that found in normal animals after the subcutaneous injection of labeled erythrocytes.

DISCUSSION

Previous studies of tumor-bearing animals have shown that radioiron from red cells injected intravascularly accumulated in the hemorrhagic areas of the tumor and only small amounts were found in the liver (*1*). In the present studies, it was observed that after the extravascular injection of labeled cells in normal rats a high percentage of the radioiron remained at the site of injection. Regardless of whether the red cells were lost by extravasation into the tumor or were injected intramuscularly, the distribution of iron was strikingly different from that found after the extravascular injection of a hemolysate of labeled cells. In the latter instance, a large percentage of the radioiron left the site of injection within the first few hours and was observed to enter other organs, particularly the liver. If the earlier observations in tumor-bearing animals (*1*) are interpreted on the basis of the findings of the present studies, they suggest that the red cells which hemorrhaged into the area of the tumor were destroyed predominantly by direct phagocytosis and not by lysis. Any such conclusion would have to be made with certain reservations as the possibility remains that in certain tumors with large necrotic centers or with massive organized clots destruction of erythrocytes by lysis may occur with subsequent trapping of their constituents.

In most previous studies on the fate of extravascular red cells, the cells have been labeled with Cr^{51}. As sodium chromate labels the globin portion of the hemoglobin molecule, the distributions of labeling would be similar to that of iron only in those instances in which the heme and globin moieties of the hemoglobin molecule remain together. Hollingsworth (*9*) measured the rate at which red cells and hemolysates labeled with Cr^{51} left extravascular sites of injection in rabbits, by the use of an external counting technique. The distribution of radiochromium from subcutaneous stroma-

free hemolysates was similar to that found in the present studies, as 90 percent of the radioactivity had disappeared within 2 days. The last 10 percent of the radiochromium appeared to be rather firmly bound to the tissue. Although the radioactivity of chromium-labeled red cells disappeared more slowly than that of the hemolysate, the rates found after the subcutaneous or intramuscular injection of chromium-labeled red cells were much faster than those observed in the present study for the iron atom from red cells.

Stohlman *et al.* (*10*), studying the radiochromium activity of blood after the intramuscular injection of 4 ml. of labeled cells into each femoral muscle of dogs, calculated that 20 percent of the red cells were returned to the general circulation over a 5-day period. These results differ from those reported here in which the total loss of activity from the injection site was only 1 or 2 percent a day, and suggest that the site and volume of the blood injected, as well as the species of animal studied, may influence the results obtained. The finding (*10*) that only 1 percent of the labeled cells injected subcutaneously entered the circulation is of interest since both the study of Hollingsworth (*9*) and the present paper indicate that the total loss of activity from the subcutaneous site is more rapid than that from the intramuscular injection. Studies in which the distribution of both iron and chromium activity from labeled cells are observed simultaneously would be of value to clarify some of these points.

REFERENCES

(*1*) GREENFIELD, R. E., GODFREY, J. E., and PRICE, V. E.: Studies on the anemia of tumor-bearing animals. I. Distribution of radioiron following the injection of labeled erythrocytes. J. Nat. Cancer Inst. 21: 641–656, 1958.

(*2*) GREENFIELD, R. E., STERLING, W. R., and PRICE, V. E.: Studies on the anemia of tumor-bearing animals. II. The mechanism of erythrocyte destruction. J. Nat. Cancer Inst. 21: 1099–1107, 1958.

(*3*) PRICE, V. E., GREENFIELD, R. E., STERLING, W. R., and MACCARDLE, R. C.: Studies on the anemia of tumor-bearing animals. III. Localization of erythrocyte iron within the tumor. J. Nat. Cancer Inst. 22: 877–884, 1959.

(*4*) GREENSTEIN, J. P.: Biochemistry of Cancer, 2d ed. New York, Academic Press, Inc., 1954, pp. 518–525.

(*5*) MAVER, M. E., GRECO, A. E., LØVTRUP, E., and DALTON, A. J.: Catheptic activities of the nuclei of normal, regenerating, and neoplastic tissues of the rat. J. Nat. Cancer Inst. 13: 687–703, 1952.

(*6*) CURTIS, M. R., and DUNNING, W. F.: Transplantable lymphosarcomata of the mesenteric lymph nodes of rats. Am. J. Cancer 40: 299–309, 1940.

(*7*) WOLLMAN, S. H., SCOW, R. O., WAGNER, B., and MORRIS, H. P.: Radioiodine uptake by transplantable tumors of the thyroid gland in C3H mice. I. Experimental results. J. Nat. Cancer Inst. 13: 785–805, 1953.

(*8*) STERLING, W. R., GREENFIELD, R. E., and PRICE, V. E.: Erythrocytes of tumor-bearing animals. (Abstract.) Fed. Proc. 17: 316, 1958.

(*9*) HOLLINGSWORTH, J. W.: Disappearance of radioactive chromium tagged erythrocytes from tissues and body cavities of rabbits. Proc. Soc. Exper. Biol. & Med. 87: 493–498, 1954.

(*10*) STOHLMAN, F., JR., BRECHER, G., SCHNEIDERMAN, M., and CRONKITE, E. P.: The hemolytic effect of ionizing radiations and its relationship to the hemorrhagic phase of radiation injury. Blood 12: 1061–1085, 1957.

Steroid Production *In Vitro* by Normal and Adrenal Tumor-Bearing Male Mice [1, 2]

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SUMMARY

Steroid synthesis *in vitro* by *normal* adrenals and transplanted corticoid-secreting adrenal tumors of mice was studied by tissue-slice incubation methods. Corticosterone was the major steroid produced by *normal* adrenal tissue, with 11β-hydroxy-Δ⁴-androstene-3,17-dione as a minor product. Adrenal-*tumor* slices synthesized several steroids, including corticosterone and 11β-hydroxy-Δ⁴-androstene-3,17-dione as the major products. After transplantation in successive hosts, corticosterone synthesis decreased, while that of 11β-hydroxy-Δ⁴-androstene-3,17-dione increased slightly, and an unidentified compound (VI) was produced in increasing quantities. Steroid formation by adrenal-tumor tissue was less efficient than by normal adrenals. In the course of successive transplantations, responsiveness to corticotropin (ACTH) was decreased and 21-hydroxylation was markedly reduced, but there was little change in 11β-hydroxylation.—J. Nat. Cancer Inst. 24: 97–107, 1960.

WHEN JESSE GREENSTEIN embarked on a career in cancer research. this discipline was crudely empirical, groping for new avenues of approach, The virus theory had proved valid for chicken tumors, but made little headway toward applicability to tumors of subhuman mammals, and none to human cancers. Chemical carcinogenesis introduced basic biochemistry into cancer research. It related chemical structure to carcinogenesis. However, it found only a limited applicability to natural cancers, and did not explain the nature of the neoplastic change. The immunologic approach proved of little promise when it was discovered that the antigenic components of neoplastic cells differed very little, if at all, from those of the hosts from which they arose. Transplantations demonstrated a basic feature of cancer cells called "autonomy" and

[1] Received for publication January 22, 1959.

[2] This investigation was supported in part by grant C-2259 from the National Cancer Institute, National Institutes of Health, Public Health Service.

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[5] The excellent technical assistance of Mr. Andrew Davis and Miss Evemarie Celozzi is gratefully acknowledged. We are indebted to Dr. Seymour Lieberman, College of Physicians and Surgeons, Columbia University, in whose laboratories the infrared analyses were carried out.

indicated that cancer cells have an individuality of their own. Characterization of cancers remained mainly descriptive, limited to appearances and behavior in the body. Much progress has been made in all these areas, but not at the time Jesse Greenstein began cancer research. In search for basic differences between cancer and normal cells, Jesse Greenstein turned to a study of their enzymes, the proximal determinants of cell function.

Pathologists, whose business has been to characterize normal cells, have gradually come around to follow his footsteps by utilization of histochemical techniques. Still, the routine of the day is to kill tissues in fixatives, promptly after receipt. A radical departure from this, pursued experimentally in our laboratories, calls for examination of the functional capacity of living cells and responsiveness to forces which normally regulate their numbers. Neoplasms related to endocrine organs are especially suited for such studies. Much is known about hormonal regulation of cell numbers and the influence of enzymes and hormones on them. Changes in enzymatic activities may explain the cancerous deviation from normal, *i.e.*, the fundamental characteristic feature of neoplastic cells called "autonomy."

Endocrine systems offer many possibilities for exploration of enzymatic disturbances. An encouraging lead, which is highly relevant to apparently unrestrained growth, was the discovery that deficiency of several hydroxylases is associated with some adrenal tumors. Lack of these enzymes causes deficiency in glucocorticoids—the physiologic inhibitor of the fasciculata layer of the adrenal cortex which secretes the glucocorticoids. The symptoms caused by these adrenal tumors result from piling up of precursor steroids with androgenic potency. Administration of cortisone will arrest the disturbance.

Having isolated numerous hormone-secreting tumors, our section has chosen them for model studies. Such tumors are now readily inducible. Hyperplastic and neoplastic monomorphous masses of these cells are readily obtainable. (Jesse Greenstein was plagued with the problem of how to get monomorphous masses of cancer and normal cells.) Furthermore, much is known now of the enzymatic capacities of endocrine cells and of their homeostasis.

Three model systems are under study in our laboratories: (*a*) thyroid and thyrotropes; (*b*) estrogens, mammotropes, and the mammary glands, and (*c*) adrenals and adrenotropes.

Bloch and Cohen chose the latter. First, they have demonstrated that experimental adrenal tumors respond to adrenotropes *in vitro*, nearly as well as normal adrenocortical cells; then they turned to the determination of the enzymatic capabilities of the neoplastic adrenal cells. As will be shown, they not only demonstrated a deviation from normal but also a further degradation of specific enzymatic capabilities, associated with greater virulence of tumor cells.

The work is fragmentary, but proceeds in a direction which will teach us how to characterize neoplastic cells, with the ultimate aim of inhibiting

them, or providing crutches for missing key functions. By defining the specific capabilities of neoplastic cells, the target for chemotherapy is pinpointed. Because of hormonal-enzymatic specificities, harm to normal cells can be avoided; destruction of endocrine organs can be readily compensated for.

This is the road Jesse Greenstein began to construct.

* * *

FUNCTIONAL adrenal tumors in mice, with androgenic or estrogenic activity, have been described by several authors (*1–6*). Hormonal activity was studied by histochemical and anatomical means; data on the endocrine chemistry of the tumors, particularly steroid production, are lacking.

Adrenal tumors, with corticoid-secreting activity, as indicated by changes in tumor-bearing hosts, were found in mice of the LAF_1 strain that had been exposed to ionizing radiation. Function was retained in tumors transplanted in successive generations of LAF_1 mice. The histological and physiological characteristics and effects of this adrenal tumor on host animals (*7*), as well as its responsiveness to corticotropin (ACTH) (*8*, *9*) have been described. This report gives the results of studies *in vitro* of steroid biosyntheses and conversions by the adrenal tumor. Comparisons were made with steroid production by normal mouse adrenals.

MATERIALS AND METHODS

Normal adrenal and adrenal-tumor slices were incubated, with and without ACTH (U.S.P. Reference Standard), by methods previously reported (*8*). In part of this study, tissue from the adrenal tumors was incubated with steroid substrates[6] in concentrations of 100 to 200 μg. (0.3–0.7 μmole) per 100 mg. of tissue. After exhaustive extractions, the combined extracts were analyzed by paper chromatography. The concentrated extracts were placed on filter-paper strips and chromatographed in the solvent system toluene-propylene glycol (*10*); the overflow was then chromatographed in the system heptane-propylene glycol (*11*). The Zimmermann (*11*), "blue tetrazolium" (BT) (*12*) reactions, and ultraviolet-light absorbency (Minerallight, Ultraviolet Products, Inc.) were employed to detect steriods. The reactive zones were eluted and used for quantitation and characterization of the steroidal material.

Concentration was determined by measurement of absorbency at 240 mμ in the Beckman Model DU spectrophotometer (Δ^4-3-ketosteroids) or by quantitative Zimmermann reactions for 17-ketosteroids.

[6] The steroids used in this study were obtained through the generosity of Drs. K. Pfister (Merck and Co.), O. Mancera (Syntax, S. A.), M. A. Sheri (Upjohn Co.), P. Perlman (Schering, Inc.), and M. Gut (Worcester Foundation for Experimental Biology).

Acetylation was carried out by adding 2 drops of anhydrous pyridine and 1 drop of acetic anhydride to one third of the dried eluate. After standing for 20 hours at room temperature, the solvents were evaporated under nitrogen at less than 40° C. and the residue was chromatographed.

Chromic acid oxidation was carried out as follows: 0.05 mg. CrO_3, dissolved in 0.05 ml. glacial acetic acid, was added to one third of the dried eluate. After 20 to 30 minutes' shaking at room temperature, 1 ml. of water was added and sufficient Na_2SO_3 to reduce excess chromic acid. The ketosteroid was extracted 3 times with 3 ml. of redistilled ethyl acetate or diethyl ether, washed with 0.1 N NaOH and water to *p*H 7, and the solvent removed as stated. The residue was then subjected to chromatography.

Sodium bismuthate oxidation was used to convert cortisol to 11β-hydroxy-Δ^4-androstene-3,17-dione, according to the procedure of Brooks and Norymberski (*13*).

RESULTS

Normal Adrenal and Adrenal-Tumor Incubations

Incubations of slices of normal mouse adrenals and of adrenal tumors were performed concurrently. Three series of tumor incubations were performed. Series A consisted of tumors from third-generation transplants. Slices of these steriod-secreting tumors were sensitive to low levels of ACTH (0.25–3.0 milliunits) as described earlier (*8*, *9*). In some of the seventh-generation transplants it was noted that the tumors were no longer stimulated by low dosages of ACTH, although linear log-dose response curves could be obtained in the range of 5 to 200 milliunits of the hormone. With maximal amounts of ACTH the total production of Δ^4-3-ketosteroids secreted by these tumor slices was similar to that found in Series A. Further incubation studies were made of this passage to determine possible steroid spectrum changes that may have occurred with successive tumor passages in conjunction with loss of tumor sensitivity to ACTH. These incubations are designated as Series B. Series C consisted of incubations from further passages of the tumor (eighth through eleventh generations).

Paper chromatographic separation of the components of the several incubation extracts yielded 4 (normal mouse adrenal incubation) to 6 (incubations A and B) ultraviolet-absorbing zones (table 1). Three of these were identified (*see* later) as 11β-hydroxyprogesterone (compound II), 11β-hydroxy-Δ^4-androstene-3,17-dione (compound III), and corticosterone (compound V), respectively; and one was tentatively designated as 11-dehydrocorticosterone (compound IV) on the basis of paper chromatographic characteristics. 17-Keto- or α-ketolic steroids, with ring A saturated, were not found.

The major steroid synthesized by *normal* mouse adrenal incubates under maximally stimulating concentrations of ACTH was corticosterone (72% of the Δ^4-3-ketosteroids); 11β-hydroxy-Δ^4-androstene-3,17-dione was pres-

TABLE 1.—Paper chromatographic characteristics of ultraviolet-absorbing compounds produced by mouse adrenal tumor and normal adrenal slices incubated with ACTH*

Compound	Occurrence†	Solvent system	Chromatographic characteristics			Identification
			Mobility‡ (*cm./hr.*)	Color reagent: Zimmermann	Color reagent: BT	
I	A, B, C, N	Heptane-propylene glycol	2.10	Gray-blue	—	—
II	A, B		0.16	Gray-blue	—	11β-Hydroxyprogesterone
III	A, B, C, N		0.10	Purple	—	11β-Hydroxy-Δ⁴-androstene-3,17-dione
			3.54	Purple	—	
IV	A, N	Toluene-propylene glycol	2.80	None	Violet	—
V	A, B, N		1.75	None	Violet	Corticosterone
VI	B, C		1.03	Gray-green	None	—
VII	A, B, C		0.055	None	None	—

*One hundred mg. tissue incubated in 3-ml. Krebs-Ringer bicarbonate buffer, *p*H 7.4, plus 6-mg. glucose and maximally stimulating amounts of ACTH for 4 hours at 37° C. in 95% O_2: 5% CO_2. Ultraviolet-absorbing zones on paper chromatograms detected by use of Mineralight lamp, maximum emission at 253 mμ.

†Incubation from third (A), seventh (B), and eighth to eleventh (C) generation transplants. Normal adrenal incubation (N).

‡Mobility of reference steroids (cm./hr.): progesterone = 2.00, 17α-hydroxyprogesterone = 0.28, 11β-hydroxy-Δ⁴-androstene-3,17-dione = 0.10 (all in heptane-propylene glycol): 11β-hydroxy-Δ⁴-androstene-3,17-dione = 3.5, corticosterone = 1.80, 11-deoxycortisol = 1.45, cortisol = 0.10 (all in toluene-propylene glycol).

ent in smaller quantities (table 2) (*8*). Adrenal *tumor*, when similarly incubated, initially (incubation A) produced approximately twice as much corticosterone as 11β-hydroxy-Δ⁴-androstene-3,17-dione (*8*). In extracts from incubations B and C, 11β-hydroxy-Δ⁴-androstene-3,17-dione and compound VI were the major incubation products; corticosterone was not detected in the extract from incubation C. In incubation A, it was noted that the synthesis of corticosterone increased with increasing ACTH concentrations, while 11β-hydroxy-Δ⁴-androstene-3,17-dione formation remained essentially unaltered.

TABLE 2.—Concentration of steroids in incubates of normal mouse adrenal and adrenal-tumor slices*

Incubation series	ACTH added (*milliunit*)	Total tissue incubated (*gm.*)	Total Δ⁴-3-KS†	Corticosterone	11β-Hydroxy-Δ⁴-androstene-3,17-dione	Compound VI
			(*μg./100 mg. adrenal/2 hours [average]*)			
			Normal adrenals			
—	0	0.09	10.4	n.d.‡	n.d.	n.d.
—	210–336	0.08	63.0	45.7	8.7	n.d.
			Adrenal tumor			
A	0	2.10	2.9	n.d.	0.4	n.d.
A	25–60	2.00	15.2	5.4	2.7	n.d.
B	40	6.40	13.7	0.3	present	present
C	40–200	16.60	15.4	n.d.	9.4	2.8

*For details of incubations, *see* table 1. ACTH concentrations added were maximally stimulating. Data in lines 1 to 4, except for compound VI, are from (*8*).

†Total Δ⁴-3-ketosteroids (absorption at 240 mμ).

‡Not detected. For normal adrenal, adrenal-tumor incubation A, B, and C, lower limits of detection on paper chromatograms were 4, 0.2, 0.08, and 0.02 μg. per 100 mg. tissue, respectively.

Adrenal-Tumor Incubations with Steroid Substrates

C_{19} steroids.—Adrenal-tumor slices, 10.2 gm., obtained from tumors of the same passage as used for incubation C, were incubated with 19.8 mg. of Δ⁴-androstene-3,17-dione. The incubation conditions and procedures for extraction and purification were identical to those described.

Total Δ⁴-3-ketosteroids, 14.6 mg., or 73 percent of the Δ⁴-androstene-3,17-dione added, was recovered. Upon separation of the components of the incubation extracts by paper chromatography, four Δ⁴-3-ketosteroids were isolated and identified (*see* later) as *Δ⁴-androstene-3,17-dione* (compound VIII, crystallized from ether-hexane, melting point—138 to 140° C. (*14*); infrared absorption spectrum; 5.20 mg.), 11β-hydroxy-Δ⁴-androstene-3,17-dione (compound III; 6.20 mg.), testosterone (compound IX; 1.59 mg.), and 11β-hydroxytestosterone (compound XI; 0.28 mg.). A fifth Δ⁴-3-ketosteroid (compound X), with a mobility identical to that of Δ⁴-androstene-3,11,17-trione and present in quantity less than 0.1 mg., was detected on the paper chromatograms.

In another series of experiments, 1.0 to 1.6 mg. of dehydroepiandrosterone was incubated with adrenal-tumor slices obtained from tumor passages used for incubation B. Forty-five to 60 percent of dehydroepiandrosterone was converted to several Δ⁴-3,17-diketosteroids, including Δ⁴-androstene-

3,17-dione, as judged by paper chromatographic criteria. Insufficient amounts precluded definitive identification.

C_{21} steroids.—Slices of adrenal-tumor tissue (incubation B) were incubated with 0.7 to 2.0 mg. of progesterone, 11-ketoprogesterone or 17α-hydroxyprogesterone (7 separate experiments). Progesterone and 11-ketoprogesterone were converted to compound II, identified as 11β-hydroxyprogesterone (*see* next section). α-Ketolic C_{21} steroids with or without 17α-hydroxylation were not detected. 17α-Hydroxyprogesterone was converted to several Δ^4-3-ketosteroids, including cortisol (compound XII; *see* next section) but not 11-deoxycortisol. Of the total Δ^4-3-ketosteroids, 10 to 20 percent was cortisol.

Identification of Compounds II, III, V, and VI

The paper chromatographic characteristics of *compound II* (*cf.* table 1) indicated the presence of a hydroxylated progesterone derivative. Reacting compound II with acetic anhydride in pyridine and rechromatography yielded unaltered compound II. The sulfuric acid absorption spectrum (*15*) of compound II, after elution from the chromatogram, showed maxima at 290 and 474 mμ, minima at 232 and 380 mμ, with an inflection at 318 mμ. These values are compatible with those of 11β-hydroxyprogesterone.

In order to confirm this identification, 80 μg. of compound II, obtained from an extract of mouse adrenal-tumor tissue incubated with progesterone and 11-ketoprogesterone (*see* previously), was oxidized with CrO_3. The oxidation product had a flow rate of 0.90 cm. per hour in heptane-propylene glycol, identical with that of crystalline 11-ketoprogesterone. The sulfuric acid absorption spectra of compound II from the two separate sources were identical. It is concluded that compound II is *11β-hydroxyprogesterone.*

Compound III exhibited chromatographic behavior suggestive of 11β-hydroxy-Δ^4-androstene-3,17-dione. It was unreactive to acetylation. The mobility of the chromic acid oxidation product in heptane-propylene glycol (0.34 cm./hr.) was essentially identical with that of Δ^4-androstene-3,11,17-trione (0.33 cm./hr.). The absorption spectrum of the reaction product of compound III with sulfuric acid was similar to that of 11β-hydroxy-Δ^4-androstene-3,17-dione; namely, $\lambda_{max.}$: 283, 380, and 460 mμ; $\lambda_{min.}$: 241, 322, and 433 mμ; and inflection at 402 mμ.

Following an incubation of mouse adrenal-tumor tissue with Δ^4-androstene-3,17-dione (as described), 6.20 mg. of compound III was isolated in crystalline form from methanol and identified as *11β-hydroxy-Δ^4-androstene-3,17-dione* by melting point (194–195° C.), sulfuric acid, and infrared absorption spectra.

Aliquots of the eluates of *compound V* from the paper chromatograms of both normal adrenal and adrenal-tumor incubation extracts were subjected to acetylation. The mobility of compound V-acetate in the solvent system heptane-propylene glycol was identical with that of corti-

costeroneacetate (0.16 cm./hr.). (Chromic acid oxidation of an aliquot of the eluate of compound V from the adrenal-tumor incubation extracts yielded a trace of Δ^4-androstene-3,11,17-trione, indicating contamination with 11β-hydroxy-Δ^4-androstene-3,17-dione.) The remaining eluates were pooled (total of 0.78 mg.) and purified by silica-gel column chromatography (10 gm. of silica-gel, 60–200 mesh, Davison Chemical Co.; 9.0 × 1.5-cm. column; solvents: 100 ml. benzene:ethyl acetate (9:1), 200 ml. benzene : ethyl acetate (3 : 1), and 100 ml. ethyl acetate). Crystalline material, 0.43 mg., obtained from the benzene : ethyl acetate (3 :1) fraction and recrystallized from diethyl ether-ethyl acetate, melted at 178 to 181.5° C. The sulfuric acid absorption spectrum ($\lambda_{max.}$: 241, 288, 331, 375, and 418 mμ; $\lambda_{min.}$: 288, 320, 365, and 405 mμ, inflection at 245 and 450–460 mμ) was similar to that of authentic corticosterone (the minima at 247 and 432 mμ and maxima at 460 mμ being masked), confirming the identification of compound V as *corticosterone*.

Compound VI, one of the two major products of incubations B and C, was unreactive to the BT reagent (indicating absence of an α-ketolic side-chain), reacted atypically with the Zimmermann reagent, formed an acetylated derivative with a mobility of 0.34 cm. per hour in the solvent system heptane-propylene glycol (suggesting the presence of two hydroxyl groups) and could not be recovered after oxidation with chromic acid. Compound VI may be identical with Southcott's compound 0.6B (*16*).

Identification of Compounds IX, XI, and XII

Compound IX, with mobility and blue Zimmermann reaction color characteristic of *testosterone*, after crystallization from ethyl acetate-hexane, melted at 151 to 153° C. Admixture with crystalline testosterone did not depress the melting point (152–153.3° C.). No separation occurred upon chromatography of crystalline testosterone or testosterone-acetate mixed with compound IX and its acetate, respectively, in the solvent system heptane-propylene glycol. Oxidation of 40 μg. of compound IX with chromic acid resulted in the formation of Δ^4-androstene-3, 17-dione as judged by paper chromatographic mobility. The infrared absorption spectrum was identical with that of testosterone.

The fourth conversion product of Δ^4-androstene-3,17-dione, *compound XI*, was more polar (0.22 cm./hr. in toluene-propylene glycol) than corticosterone (0.38 cm./hr.), did not react with BT reagent, and gave a blue color with the Zimmermann reagent. Mixed chromatography of compound XI with authentic 11β-hydroxytestosterone in heptane:toluene (1:1)/propylene glycol yielded a single zone (0.036 cm./hr.). Acetylation and chromic acid oxidation of both the free and acetylated compound resulted in derivatives with mobilities identical to 17-acetoxy-11β-hydroxy-Δ^4-androstene-3-one, Δ^4-androstene-3,11,17-trione and 17-acetoxy-Δ^4-androstene-3,11-dione, respectively. These analytical data are compatible with identification of compound XI as *11β-hydroxytestosterone*.

Compound XII, obtained from the incubation of 17α-hydroxyprogesterone with adrenal-tumor slices, was identified as *cortisol* by paper chromatographic techniques. Mobility in toluene-propylene glycol of free (0.10 cm./hr.) and acetylated (1.00 cm./hr.) compound XII was identical with that of crystalline reference standards of cortisol. Oxidation with chromic acid and sodium bismuthate, respectively, converted compound XII to Δ^4-androstene-3,11,17-trione and 11β-hydroxy-Δ^4-androstene-3,17-dione, identification resting on comparative chromatographic mobility in heptane-propylene glycol.

DISCUSSION

Corticosterone was the major secretory product synthesized by the adrenals of *normal* mice *in vitro*. This is in agreement with findings of Hofmann (*17*) and Southcott *et al.* (*16*). Wilson and coworkers (*18*) tentatively identified corticosterone from serum and 11β-hydroxy-Δ^4-androstene-3,17-dione and 11β-hydroxyandrostene-3,17-dione from urine of the same strain of mice, as used by us, bearing transplantable ACTH-secreting pituitary tumors. In our experiments *in vitro* corticosterone constituted 72 percent of the total Δ^4-3-ketosteroids, which is in the general range of α-ketolic steroid percentages noted with most species (*19*). 11β-Hydroxy-Δ^4-androstene-3,17-dione was a minor product (about 20%), with trace amounts of two other steroids. In contrast to normal adrenals, steroidogenesis by the adrenal *tumor* was characterized by decreased absolute steroid synthesis per unit tissue weight and greater diversity of major endproducts. Furthermore, there were quantitative changes in steroid production with successive tumor transplantations coupled with diminished sensitivity to ACTH and increased virulence of tumor. C_{19}-steroid production in the form of 11β-hydroxy-Δ^4-androstene-3,17-dione constituted an increasing percentage of the total Δ^4-3-ketosteroids with successive transplantations.

The adrenal tumor produced progressively less BT reactive steroids after successive transplantation. This observation may be explained on the basis of: (*a*) decreased 21-hydroxylase activity, (*b*) increased reduction of the C-20 carbonyl group, or (*c*) increased C_{19}-steroid production at the expense of C_{21}-steroids. The absence of paper chromatographic zones corresponding to the 20-hydroxylated corticosterone derivatives speaks against increased 20-hydroxysteroid dehydrogenase activity. Incubation of adrenal-tumor slices with either corticosterone or cortisol did not yield any 20-hydroxy derivatives. The data do not allow a choice between the other two explanations, particularly because of the presence of an unidentified compound (VI). 3β-Hydroxysteroid dehydrogenase, Δ^4-isomerase and 11β-hydroxylase activity remained essentially unchanged in the course of numerous successive tumor transplantations. 17α-Hydroxycorticoids were not formed by either the normal or tumor tissue, which is in accord with the observation by Hofmann (*17*) that mice are an extreme

example of species with markedly diminished ability to form 17α-hydroxy-corticoids. Further, 17α-hydroxyprogesterone was not converted to 17-ketosteroids. In this connection, it is of interest to raise the question whether sufficient 17α-hydroxylated precursors of 11β-hydroxy-Δ^4-androstene-3,17-dione are formed to explain the appreciable endogenous formation of this C_{19}-steroid or whether another pathway is involved.

The results of the incubation experiments with steroid substrate confirm the ability of the adrenal tumor to synthesize 11β-hydroxylated C_{19}- and C_{21}-Δ^4-3-ketosteroids from $C_{19}O_2$ (*i.e.*, dehydroepiandrosterone or Δ^4-androstene-3,17-dione) or $C_{21}O_2$ (*i.e.*, progesterone) precursors. 17α-Hydroxylase activity was absent and 21-hydroxylase activity was progressively reduced. The conversion of 17α-hydroxyprogesterone to cortisol was relatively low, and 11-deoxycortisol was not detected. Similarly, neither progesterone nor 11-ketoprogesterone was converted to detectable amounts (> 2 %) of corticosterone or 11-dehydrocorticosterone.

The reduction of Δ^4-androstene-3,17-dione to testosterone is of interest. Testosterone (or other 17β-hydroxy-C_{19}-steroids) has only been detected in human adrenal tumor but not in normal adrenal tissue (*20*).

The factors underlying the changes in tumor steroid synthesis with successive transplantation are not clear. Deviations from normal steroid pathways in human endocrine tumors have been observed by several investigators (*21–26*). It is well known that tumors undergo changes in the course of successive transplantation. The usual event is that of diminution of specific functions; the best explanation for this is the loss of specific enzymatic capacities due to heritable modifications (probably somatic mutations). The very essence of tumorous transformation of normal cells is, according to the widely held theory of Potter (*27*), that of enzyme deletion. It is suggested that the study of steroidogenic function of adrenal-tumor tissue may lead to better understanding of tumor growth of adrenal cells.

REFERENCES

(*1*) Woolley, G. W., Fekete, E., and Little, C. C.: Mammary tumor development in mice ovariectomized at birth. Proc. Nat. Acad. Sc. 25: 277–279, 1939.

(*2*) Gardner, W. U.: Estrogenic effects of adrenal tumors of ovariectomized mice. Cancer Res. 1: 632–637, 1941.

(*3*) Woolley, G. W., and Little, C. C.: The incidence of adrenal cortical carcinoma in gonadectomized female mice of the extreme dilution strain. II. Observations on the accessory sex organs. Cancer Res. 5: 203–210, 1945.

(*4*) ———: The incidence of adrenal cortical carcinoma in gonadectomized male mice of the extreme dilution strain. Cancer Res. 5: 211–219, 1945.

(*5*) Kirschbaum, A., Frantz, M., and Williams, W. L.: Neoplasms of the adrenal cortex in noncastrate mice. Cancer Res. 6: 707–711, 1946.

(*6*) Frantz, M. J., and Kirschbaum, A.: Sex hormone secretion by tumors of the adrenal cortex of mice. Cancer Res. 9: 257–266, 1949.

(*7*) Cohen, A. I., Furth, J., and Buffett, R. F.: Histological and physiological characteristics of hormone-secreting transplantable adrenal tumors in mice and rats. Am. J. Path. 23: 631–651, 1957.

(8) COHEN, A. I., BLOCH, E., and CELOZZI, E.: *In vitro* response of functional experimental adrenal tumors to corticotropin (ACTH). Proc. Soc. Exper. Biol. & Med. 95: 304–309, 1957.

(9) COHEN, A. I., and FURTH, J.: Corticotropin assay with transplantable adrenocortical tumor slices: Application to the assay of adrenotropic pituitary tumors. Cancer Res. 19: 72–78, 1959.

(10) BURTON, R. B., ZAFFARONI, A., and KEUTMANN, E. H.: Corticosteroids in urine of normal persons determined by paper chromatography. J. Biol. Chem. 193: 769–779, 1951.

(11) SAVARD, K.: Paper partition chromatography of C_{19}- and C_{21}-ketosteroids. J. Biol. Chem. 202: 457–477, 1953.

(12) CHEN, C., and TEWELL, H. E., JR.: Corticosteroid determination with blue tetrazolium BT. (Abstract.) Fed. Proc. 10: 377–378, 1951.

(13) BROOKS, C. J. W., and NORYMBERSKI, J. K.: The oxidation of corticosteroids with sodium bismuthate. Biochem. J. 55: 371–378, 1953.

(14) DAVID, K.: Über das Testosteron, das kristallisierte männliche Hormon aus Stierentestes. (Testosterone, the crystallized male hormone from steer testes. I.) Acta brev. Neerland. 5: 85–86, 1935.

(15) ZAFFARONI, A.: Absorption spectra of sulfuric acid chromogens obtained from adrenal steroids and related compounds. J. Am. Chem. Soc. 72: 3828, 1950.

(16) SOUTHCOTT, C. M., BANDY, H. E., NEWSOM, S. E., and DARRACH, M.: Metabolism of adrenal steroids in the mouse. I. Observations on 20 α-dihydrocorticosterone and corticosterone in the plasma of mice treated with corticotropin. Canad. J. Biochem. & Physiol. 34: 913–918,1956.

(17) HOFMANN, F. G.: Observations on *in vitro* adrenal steroid synthesis in the albino mouse. Endocrinology 59: 712–715, 1956.

(18) WILSON, H., BORRIS, J. J., and BAHN, R. C.: Steroids in the blood and urine of female mice bearing an ACTH-producing pituitary tumor. Endocrinology 62: 135–149, 1958.

(19) BUSH, I. E.: Species differences in adrenocortical secretion. J. Endocrinol. 9: 95–100, 1953.

(20) ANLIKER, R., ROHR, O., and MARTI, M.: Untersuchungen über Organextrakte. Über den Nachweis von Testosteron in einem virilisierenden Nebennierenrindentumor. Helv. chim. acta 39: 1100–1106, 1956.

(21) TOUCHSTONE, J. C., GLAZER, L., COOPER, D. Y., and ROBERTS, J. M.: The isolation of Δ^4-androstene-11β-ol-3,17-dione from human adrenal incubates. J. Clin. Endocrinol. and metabolism 15: 382–383, 1955.

(22) BERLINER, M. L., BERLINER, D. L., and DOUGHERTY, T. F.: Metabolism of progesterone by adrenal tissue from patients with Cushing's syndrome and mammary carcinoma. J. Clin. Endocrinol. and metabolism 18: 109–114, 1958.

(23) SALHANICK, H. A., and BERLINER, D. L.: Isolation of steroids from a feminizing adrenal carcinoma. J. Biol. Chem. 227: 583–590, 1957.

(24) GALLAGHER, T. F., KAPPAS, A., SPENCER, H., and LASZLO, D.: Influence of invasiveness, hormones, and amphenone on steroids in adrenal carcinoma. Science 124: 487–489, 1956.

(25) SAVARD, K., DORFMAN, R. I., BAGGETT, B., and ENGEL, L. L.: Steroid 11β-hydroxylation by a human testicular tumor. (Abstract.) Fed. Proc. 15: 346–347, 1956.

(26) ENGEL, L. L., DORFMAN, R. I., and ABARBANEL, A. R.: Neutral steroids in the urine of a patient with a luteoma of the ovary. J. Clin. Endocrinol. 13: 903–910, 1953.

(27) POTTER, V. R.: The present status of the deletion hypothesis. Univ. Michigan M. Bull. 23: 401–412, 1957.

On the Carcinogenicity of an Iron-Dextran Complex [1, 2]

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SUMMARY

A recent observation, by H. G. Richmond, on the carcinogenicity of an iron-dextran complex, is confirmed and extended, the complex having proved markedly carcinogenic in the rat and mouse after subcutaneous injection of massive doses. The bulk of tumors appear at the site of injection as sarcomas arising from the iron-laden connective and reticuloendothelial tissues. The component dextran is inactive. It is still not known whether the potency of the complex is a function of the metal alone or of the entire complex. The various possibilities are considered, with particular reference to alternative mechanisms of action involving differing aspects of iron metabolism, and including the inactivation of vitamin E.—J. Nat. Cancer Inst. 24: 109–147, 1960.

THE GREAT range of chemicals capable of exerting carcinogenic effects has long been known to embrace specific metals and metalloids, *e.g.*, arsenic, beryllium, chromium, cobalt, nickel, and zinc—including some of the relatively common members of the transition elements. In most cases such action has been demonstrated only in somewhat limited or special conditions. Particularly noteworthy is the involvement of various metals and minerals—chromium, nickel, iron oxide, radioactive metals, arsenic, asbestos, and, possibly, beryllium—in the production of cancer of the respiratory tract, including the lung of man (*1*). In many such instances, certainly in the field of industrial toxicology, a difficulty is

[1] Received for publication October 9, 1959.

[2] The investigation in general has been supported by grants from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] With the technical assistance of B. C. V. Mitchley and K. G. Moreman.

[4] The authors desire to acknowledge many helpful discussions with Professors F. Bergel and E. Boyland; Drs. L. A. Elson, L. Golberg, E. F. Hartree, and H. G. Richmond, and Mr. G. M. Timmis; and the valuable assistance of Mr. J. L. Everett. Many of the preparations were provided by Benger Laboratories, Ltd., through the kindness of Mr. B. D. Thornley, Dr. L. Golberg, and Mr. F. Fowler; the company also made a special grant toward the costs of this investigation. Various phthalocyanines were given by Dr. J. A. Elvidge, and other compounds by Imperial Chemical Industries, Ltd., and by Dr. M. W. Partridge. We are also grateful to Dr. E. H. Mercer and Mr. M. S. C. Birbeck for their assistance in electron microscopy, and to Mr. B. C. V. Mitchley, Mr. A. Green, and Mr. N. Casperd for technical assistance. The photography is the work of Mr. K. G. Moreman and his department.

encountered in that the suspected product may represent a complex mixture: for example, nickel ore may contain amounts of almost every other metal, including copper, cobalt, and the precious elements. In the list of metals, iron has not hitherto drawn special attention, though Argyll Campbell (*2*) studied the influence of iron oxide, in conjunction with precipitated silica, on the incidence of primary lung tumors in mice. Faulds and Stewart (*3*) reported that in 1948–53, 15 percent of 89 consecutive autopsies of hematite miners showed cancer of the lung, and that the tumors were usually located in areas of fibrosis due to siderosilicosis. Further, Bonser, Faulds, and Stewart (*4*) believed that silica might be the common factor in the environmental exposure of the asbestos worker and the hematite miner, that fibrosis precedes malignant change in the lung, and that both the asbestos and the hematite (iron oxide) might somehow convert the fibrogenic influence of silica into a carcinogenic action. It is, however, possible that iron oxide itself may possess greater relevance, than has been thought hitherto, as a factor common to the hazard in the chromate, hematite, nickel, and asbestos industries.

However, the whole subject has received a new stimulus through an unexpected observation recently made by Richmond (*5–7*) [*see also* Haddow (*8*)] on the carcinogenicity of an iron-dextran complex, which it is the purpose of the present paper to confirm and extend. The observation not merely has great interest in its own right, but it also carries, as it is hoped to show, many implications of potential importance, and has already prompted many new ideas and experiments. On both these grounds it appeared appropriate as the subject of a paper devoted to the memory of Jesse Greenstein, bearing in mind not only his contributions to the biochemistry of carcinogenesis in general, but also his special interests in the metal enzymes, in the role of iron, and especially in catalase.

In studies of the effects of repeated injection of trypan blue in the rat, in inducing pleomorphic histiocytic tumors, as had earlier been shown by Gillman and Gillman (*9*), Richmond wished to employ a "negative control," that is, a substance which, equally with trypan blue, would be taken up by the reticuloendothelium, but without producing tumors. Since the possibility that iron had any part in carcinogenesis had scarcely been entertained, and since large amounts of iron may accumulate in the body in a variety of conditions, such as hemochromatosis and transfusional, malnutritional, or occupational siderosis, Richmond chose as his control, in the expectation that iron must be noncarcinogenic if not altogether bland, the iron-dextran complex Imferon (Benger Laboratories, Ltd.). [This colloidal preparation was the subject of British Patent Specification 748,024 (London and Twigg), April 18, 1956.] Imferon is a sterile solution in water of a complex of ferric hydroxide and a low-molecular-weight dextran fraction. Each 2 ml. of the solution contains 100 mg. of iron, calculated as Fe, present as ferric hydroxide in complex—the amount of dextran being about 20 percent weight per volume. The solution also contains 0.9 percent sodium chloride. Estimation of the Number Aver-

age Molecular Weight [5] of dextran leads to a figure of approximately 2,500, corresponding to a chain of 15 anhydroglucose units. However, in electron micrographs (fig. 1) the particles may appear in large clusters; the true micellar and chain structure is not completely known, though X-ray diffraction and other studies (by Dr. J. Iball) are proceeding. Meantime, on account of its ready absorption from the intramuscular site, its low toxicity (*10*) (with an LD50 of 600–1200 mg.Fe/kg. by the intravenous route in mice, about one third that of saccharated oxide of iron), and its stability in the presence of proteins and electrolytes, it had found a wide application in the treatment of human iron-deficiency anemias, including those of pregnancy and rheumatoid arthritis (*11*), and of piglet anemia in veterinary practice (*12–15*). The British and American clinical literature is already extensive. A proportion of papers allude to undesirable side-effects, and the *British Medical Journal*, in 1954 (*16*), especially referred to the hazard of systemic allergic reactions when metals are administered either intramuscularly or intravenously, and believed that "It is therefore too early yet to advocate that the uncomplicated iron-deficiency anaemia so often seen in pregnancy should be treated by iron intramuscularly." In experimental siderosis induced by administration of the complex, Nissim (*17*; *see also 18, 19*) observed deposition of iron in the testes of mice, with resultant degeneration and atrophy of the seminiferous tubules. Extensive pharmacological studies of the factors in the local absorption and general distribution of such iron-polysaccharide complexes have been carried out by Golberg and his colleagues, who have also utilized the relatively low immediate toxicity of iron-dextran to investigate the effects of excessive administration and massive overload (*20–23; see also 24, 25*). The substance has further been used in intravital techniques for the demonstration of lymphatics (*26*) and in the determination of plasma volumes (*27*).

Surprisingly, Richmond found the iron-dextran complex itself to be carcinogenic, observing 37 local sarcomas in a total of 64 rats given the complex by repeated intramuscular injection, with a latent period of 10 to 14 months in adult rats and about 9 months in young rats. The early results were reported in a communication to the Pathological Society in January 1957. For several reasons it appeared desirable to test the complex in other species, and it is the purpose of this paper to confirm Richmond's discovery, to show that Imferon is a potent carcinogen in both the rat and mouse at least, certainly under the conditions employed, and lastly to consider the bearing which these facts may possess, upon the problems of carcinogenesis as a whole.

EXPERIMENTAL

The experiments so far complete and to be described were designed to test the carcinogenicity of iron-dextran in the mouse, rat, guinea pig, golden and cream hamsters, Chinese hamster, and rabbit. In parallel,

[5] Kindly provided by Dr. B. Ingelman, Pharmacia, Uppsala.

tests were also carried out in mice—in attempts to probe the mechanism of action—of the following agents: (1) the dextran component alone; (2) metallic sponge iron, dialyzed iron (Fe_2O_3 5%), various iron salts, *viz.*, ferric citrate, ferric salicylate and the ascorbate, ferrous sulfate, ferrous lactate, and ferrous gluconate, and other iron-containing substances or preparations such as ferrocene, ferritin, and ferrivenin (saccharated oxide of iron); (3) other metal complexes, *viz.*, bismuth-dextran, chromium-dextran, aluminium-dextran, and copper-dextran; (4) a range of chelating and related agents, *viz.*, 8-hydroxyquinoline, an 8-hydroxyquinoline copper complex, quinisatin oxine, catechol-3,5-disulfonic acid (Tiron), Versene, Versonal (Fe^3-specific), $\alpha\alpha$-dipyridyl, phenolphthalein phosphate, and $\beta\beta$-dimethylcysteine (DL-penicillamine); (5) in the same general connection, adenine, adenosine-5-phosphate, guanylic acid, and xanthine; (6) cobalt, zinc, and nickel octamethyltetrazaporphyrins, zinc tetrabenzporphyrin, and a cross-conjugated macrocycle copper porphyrin; (7) and finally, aluminium, tin, copper, chromium and hydroxymanganese phthalocyanines, and copper and chromium phthalocyanine tetrasulfonic acids.

All preparations were administered by repeated subcutaneous injection in the flank at weekly intervals, except in the case of an Imferon preparation given by forced-feeding to the rat, and of the administration of Imferon by intramuscular injection in the hind limb of the rabbit.

Iron-Dextran Complex (Imferon)

In a pilot or orienting experiment, 25 mice received 0.2 cc. Imferon (that is, the equivalent of 10 mg. Fe) subcutaneously in the right flank at approximately weekly intervals on 13 occasions. Before proceeding, it should be emphasized that such amounts represent, in relation to body iron, a swamping dose or overload both locally and systemically, as was evident from the histochemical demonstration of iron in the liver, spleen, kidney, testis, pancreas, intestine, pituitary, lymph nodes, and other sites. The relevance of this systemic effect, bearing in mind that the "clinical dose" for mice would be only one hundredth of that used in this experiment, will be further discussed.

The first observation made in this experiment was of the appearance in the treated mice, after some months, of a considerable area of epilation at the injection site (fig. 2). At 11 months from the beginning of the experiment, 1 mouse was found to have a tumor arising locally, which proved to be a spindle-cell sarcoma with frequent mitoses (fig. 3). Both the phagocytic cells of the tumor, and many of the malignant cells themselves, contained a pigment resembling hemosiderin, and evidently consisting of loosely bound iron, from the positive Prussian-blue reaction (fig. 6). In the following 6 months, that is, 18 months from the commencement, a total of 9 similar sarcomas developed in the 14 mice surviving at 1 year (*e.g.*, figs. 4, 5, and 7). This small-scale experiment appeared, therefore, to establish the carcinogenicity of Imferon for mice, under the conditions described. It also provided three other impres-

sions: namely, the rapidity of growth and progression of these tumors; their tendency to metastasize; and the propensity to the development not only of sarcomas at the injection site but also, in some cases, of proliferative lesions and malignant tumors in distant organs. In one mouse bearing a local sarcoma (fig. 10) one lobe of the liver contained hypertrophic areas (fig. 8), probably compensatory to liver damage rather than a true hepatoma. Another developed not only a sarcoma at the site of injection, and a tumor mass (in a hind limb) which proved to be secondary, but also a primary bronchogenic carcinoma—a tumor only rarely seen in mice (figs. 11, 12, and 13). Advantage has been taken of the favorable properties of iron in electron microscopic studies. In electron micrographs of several of the tumors described here, Dr. E. H. Mercer and Mr. M. S. C. Birbeck show islands of ferritin granules in the cytoplasm (fig. 14): the appearances are in many ways reminiscent of those seen under physiological conditions in certain organs in the process of aging, for example, in the macrophages of the spleen (fig. 15). According to Richter (*28*), who has studied the cellular transformation of injected colloidal iron complexes into ferritin and hemosiderin, the physical state of the ferric hydroxide micelles contained in iron-dextran, as judged by electron microscopy and electron diffraction, differs notably from the state of the ferric hydroxide in ferritin or hemosiderin. His findings show that the iron preparations used in therapy can be identified within cells, and that their intracellular disposition and fate can be followed at the molecular level.

In a second experiment, 30 stock mice received 0.3 cc. of iron-dextran by subcutaneous injection at weekly intervals for 7 months, *i.e.*, received a much higher individual and total dose than in the first experiment. Twenty-three of the mice survived 6 months, and of these, 18 developed sarcomas at the injection site, 8 of them with latent periods under 1 year (1 at 6 months, 3 at 8 months, 3 at 10 months, and 1 at 11 months). Hence, it would appear that larger and more protracted dosage materially accelerated tumor emergence. Fifteen of the primary tumors were transplanted, with successful transmission in 9. Figure 9 shows an example of one such primary sarcoma. In others, a tendency was observed, in sections including the skin, to the formation of masses or processes of enlarged and somewhat irregular basal epithelial cells, taking on a downward growth (figs. 16 and 17) and suggesting an influence, whether direct or indirect, on epithelium as well as the connective tissues. Throughout many of the sarcomas was noted a dense concentration of pigment-laden histiocytes, many of them multinucleate, but whether the iron-dextran had induced these histiocytes to divide, or merely to mobilize, proved difficult to ascertain. However, 3 tumors were recorded (additional to the sarcomas) which could be regarded as frank histiocytomas (fig. 20). One mouse showed liver damage, hepatoma, and hemangioma; in this, as in many of the livers examined, a prominent feature was the occurrence of spherical iron-containing inclusion bodies in the nuclei of the parenchymal cells (fig. 21).

Thus far, the iron-dextran used was the clinical preparation, with the property of efficient absorption—although the conditions of these experiments, as has been made clear, of necessity produced an iron overload both locally and systemically. However, two further experiments were conducted using laboratory preparations of Imferon, known to be poorly absorbed, which were provided by Benger Laboratories, Ltd. (batches 145Z and 107/59). In the first, on 11 weekly occasions, 20 stock mice received 0.2 cc. by subcutaneous injection. Of these, 19 survived to 6 months and 7 to 1 year. Tumors were recorded in 10, including an epithelioma of the skin at the injection site after a latent period of 6½ months (figs. 18 and 19), 7 sarcomas or fibrosarcomas with latent periods of 9 to 11 months (fig. 24), a fibroma at 11 months, and an intra-abdominal lymphosarcoma at 10 months (fig. 25). Of the sarcomas, 4 grew successfully on transplantation. In the second experiment with poorly absorbed material, 20 stock mice or their survivors (13 at 8 months) received 0.2 cc. of batch 107/59 by weekly subcutaneous injection over 7½ months. Of these, 7 developed tumors locally: 4 sarcomas, and 3 histiocytomas, the latter with latent periods of 5 months and the former of 7 months and upwards.

Imferon was also tested in a series of 30 male rats of the Chester Beatty inbred albino strain, which received 1 cc., weekly for 6 months, by subcutaneous injection in the flank. For convenience, injection was made into an area previously epilated. It was soon observed that regrowth of hair was greatly retarded or almost entirely inhibited, although a tuft of new hair frequently appeared at the site of subsequent injection, no doubt stimulated by trauma (fig. 26). All 30 of the treated rats were alive at 6 months, and, thereafter, yielded a total of 24 primary tumors, 19 sarcomas or fibrosarcomas, and 5 histiocytomas, several doubtless benign (figs. 22 and 29). Transplantation was attempted in 10 sarcomas, with success in 3. Generally speaking, the results of this experiment are similar to, and fully confirmatory of, those obtained by Richmond. In addition, 20 male rats received an oral Imferon solution (Benger's, batch 10163/1, 5% w/v Fe), 1 cc. being administered by forced-feeding weekly for 8 months, while 20 mice received 0.3 cc. of this preparation in the same way. These experiments have remained negative, except for a tumor in the right groin of 1 animal, which proved to be an adenocarcinoma of the mammary gland (fig. 27) and which showed a positive Prussian-blue reaction for iron. If this isolated tumor cannot with any assurance be attributed to treatment, nevertheless the finding is perhaps suggestive, since no tumor of this type has hitherto been observed to occur spontaneously in a male rat of this strain and must therefore be excessively rare. The distribution of iron in the intestinal mucosa of the same animal is shown in figure 23.

Among other species, 20 male cream hamsters received 0.5 cc. of iron-dextran subcutaneously each week for 10 weeks; only 1 developed a sarcoma, highly pleomorphic, after a latent period of 9 months. No tumors were recorded in a second series of 30 hamsters similarly treated. Two series of 12 and 20 Chinese hamsters received doses of 0.1 and 0.3 cc.

Imferon for 7 and 4 months, respectively, with relatively high mortality, a factor in which was an extraordinary frequency of damage to and hypertrophy of the liver (fig. 28), with hyperplastic foci and, in some cases, cholangioma and hepatoma. No tumors were observed at the site of injection. Two groups of 8 and 10 guinea pigs received 1 cc. of Imferon subcutaneously each week for 7 months. Treatment was poorly tolerated; only 5 and 3 animals survived 1 year, and no tumors were recorded. Six rabbits also received 28 weekly injections of 2 cc. of Imferon, in this experiment intramuscularly, but without evident pathology after 16 months.

The Component Dextran

Two experiments were conducted in groups of 30 and 20 stock mice to test the component dextran in 20 percent solution weight per volume (batches 10150/1 and 10164/1, Benger Laboratories, Ltd.). In both, weekly doses of 0.2 cc. were administered subcutaneously for 16 and 11 months; 12 and 16 animals survived in the respective groups. No tumors were recorded in either series.

Metals, Chelating Agents, and Metal Complexes

A range of metals, chelating agents of different kinds, and metal-containing complexes (listed above) was examined in order first to test the specificity of the carcinogenic effects of iron-dextran, and secondly in the hope of clarifying the mechanism of its action. The outcome of these experiments to date is summarized in table 1. While many are still in progress, a great contrast is already clearly apparent, between the carcinogenic potency of iron-dextran, and the inactivity or relatively low activity of most of these agents. Only one sarcoma and one benign tumor were obtained with aluminium-dextran, and none with the bismuth, chromium, or copper complexes, though it appears (private communication from Dr. L. Golberg) that these metal complexes are not at all comparable with Imferon, since they are not stably chelated, and hence liberate ionic metal on injection. The occurrence of an exceedingly low (apparent) yield of tumors with ferrous sulfate, lactate, and gluconate may be of interest, though probably more significant are the few tumors attributable to ferrivenin, which substance, however, Richmond found inactive. In short, these experiments have served to set out in sharp relief the altogether higher order of carcinogenic potency of the iron-dextran complex in the mouse and rat, under the conditions described.

DISCUSSION

It is still not certain whether this outstanding carcinogenic potency of the iron-dextran complex is a function of the metal alone or of the entire complex (which undoubtedly affords an exceptionally efficient mode of iron administration), or whether a special role is played by the

TABLE 1.—Activity of metals, chelating agents, and metal-containing complexes

Agent under test	Number and strain of mice	Number of weekly subcutaneous injections, and dose	Months of experiment to date and survivors	Tumors recorded
Metallic sponge iron	20 CBA	16 20 mg.	15 (16)	Nil
Dialyzed iron (Fe_2O_3 5%).	20 CBA	16 0.2 cc.	15 (19)	Nil
Ferric citrate	20 Stock	33 5 mg.	9 (13)	Nil
Ferric salicylate	20 Stock	36 5 mg.	12 (14)	Nil
Iron ascorbate	20 Stock	43 0.2 cc. ≡0.3 mg. Fe	12 (11)	Nil
Ferrous sulfate	20 Stock	16 2.5 mg.	14 (13)	1 Fibroma
Ferrous lactate	20 Stock	21 5 mg.	12 (11)	1 Sarcoma
Ferrous gluconate	20 Stock	13 5 mg.	12 (12)	1 Fibroma 1 Thymoma 1 Thecoma
Ferrocene	20 Stock	28 5 mg.	9 (17)	Nil
Ferritin (rat)	20 (Albino rats)	10 20 mg.	7 (20)	Nil
	20 Stock (mice)	15 5 mg.	7 (12)	
Ferrivenin (saccharated oxide of iron).	20 Stock	13 0.2 cc.	14 (11)	2 Spindle-cell sarcomas 3 Histiocytomas
Bismuth-dextran (batch 10186/1, 0.5% Bi).	20 Stock	23 0.2 cc.	10 (14)	Nil
Chromium-dextran (batch 6459/1).	20 Stock	24 0.2 cc.	9 (16)	Nil
Aluminium-dextran (batch 6438/7).	20 Stock	14 0.2 cc.	14 (13)	1 Sarcoma 1 Benign histiocytoma
Copper-dextran (batch 11931/4).	20 Stock	6 0.1 cc. of 1 in 4 dilution	10 (13)	Nil
8-Hydroxyquinoline	20 Stock	31 1 mg.	15 (17)	1 Hepatoma?
8-Hydroxyquinoline copper complex.	20 Stock	39 0.1 mg.	10 (14)	1 Pleomorphic sarcoma (*see* fig. 30)
Quinisatin oxine	20 Stock	41 1 mg.	9½ (19)	Nil
Catechol-3, 5-disulphonic acid (Tiron).	20 Stock	41 5 mg.	12 (13)	1 Thymoma 1 Lymphoma
Versene	20 CBA	21 0.2 cc. of 5% solution	14 (18)	Nil
Versonal Fe^3-specific	20 Stock	46 0.2 cc.	12 (17)	Nil
$\alpha\alpha$-Dipyridyl	20 Stock	40 5 mg.	10 (12)	1 Parotid tumor
Phenolphthalein phosphate.	20 Stock	13 0.2 cc.	11 (10)	Nil
$\beta\beta$-Dimethylcysteine (DL-penicillamine).	20 CBA	19 2 mg.	13 (18)	Nil
Adenine	20 CBA	28 5 mg.	14 (15)	Nil
Adenosine-5-phosphate	20 Stock	17 5 mg.	14 (19)	Nil
Guanylic acid (sodium salt).	20 Stock	25 5 mg.	14 (15)	Nil
	20 CBA	28 5 mg.	15 (18)	Nil
Xanthine	12 (Albino rats)	18 50 mg.	14 (12)	1 Sarcoma

TABLE 1.—Activity of metals, chelating agents, and metal-containing complexes —Continued

Agent under test	Number and strain of mice	Number of weekly subcutaneous injections, and dose	Months of experiment to date and survivors	Tumors recorded
Cobalt octamethyltetrazaporphyrin.	20 Stock	8 0.5 mg.	8 (20)	Nil
Zinc octamethyltetrazaporphyrin.	20 Stock	7 0.5 mg.	8 (17)	Nil
Nickel octamethyltetrazaporphyrin.	20 Stock	5 0.5 mg.	8 (12)	Nil
Zinc tetrabenzporphyrin.	20 Stock	8 0.5 mg.	9 (18)	Nil
Cross-conjugated macrocycle copper porphyrin.	20 Stock	4 0.5 mg.	10 (14)	Nil
Aluminium phthalocyanine.	20 Stock	11 0.5 mg.	8 (17)	Nil
Tin phthalocyanine...	20 Stock	34 0.5 mg.	8 (14)	Nil
Copper phthalocyanine	20 Stock	34 0.5 mg.	8 (17)	Nil
Chromium phthalocyanine.	20 Stock	11 0.5 mg.	8 (18)	Nil
Hydroxymanganese phthalocyanine.	20 Stock	8 0.5 mg.	9 (17)	Nil
Copper phthalocyanine tetra-3-sulfonic acid.	20 Stock	36 0.5 mg.	8 (20)	Nil
Copper phthalocyanine tetra-4-sulfonic acid.	20 Stock	25 0.5 mg.	8 (11)	Nil
Chromium phthalocyanine tetra-4-sulfonic acid.	20 Stock	23 0.5 mg.	8 (16)	Nil

component dextran. The possibility of some unsuspected contaminant in the fermentative production of the dextran appears very remote (private communication, Mr. B. D. Thornley) and can be excluded with confidence. As to the dextran itself, it must be recalled that other macromolecules, such as carboxymethylcellulose and polyvinylpyrrolidone, have also been incriminated as carcinogens (*29*). More recently, Hueper (*30*) has described the carcinogenicity of some but not all dextrans, also of polyvinylpyrrolidone and polyvinylalcohol, on parenteral inoculation in rats and mice. In these experiments, however, sarcomas were observed in those organs and tissues (such as the reticuloendothelium) in which the agents were stored, and not at the site of injection. The phenomenon would therefore appear to be different in nature from that considered here. Further, Lusky and Nelson (*31*) obtained negative results on testing a particular dextran. Finally, the negative outcome of the experiments now recorded in respect to the dextran of low-molecular weight used in the preparation of the iron complex, agrees with the experience of Richmond. Hence, present indications tend to exclude the carbohydrate component of iron-dextran, at least *per se*, as responsible for the carcinogenicity of the complex.

The present experiments show that the mere administration of iron itself in various forms, though not entirely negative, is certainly insufficient to account for the overwhelming carcinogenicity of iron-dextran; and hence it is necessary to consider whether this activity is dependent on

the complex in its entirety. If this were so, the complex could operate in one of two ways: first, as a colloid that produces reticuloendothelial blockade and damage, and perhaps interference with, or even abrogation of, immune processes; and, second, as a peculiarly favorable means of introducing iron within the cell. It is clear that the role of the metal in the former mechanism could well be nonspecific or indifferent, but specific in the latter. Considering the former, it is of interest that the cellular uptake of iron-dextran is similar in its distribution to that of various antigens (*e.g.*, γ-globulin) and viruses (*e.g.*, influenza virus) in the Kupffer and reticuloendothelial cells. As to the site of action, information of some interest has been obtained by Dr. and Mrs. O. G. Fahmy, from experiments to test the mutagenicity of iron-dextran in *Drosophila*. The product was administered by intra-abdominal injection into adult males of various doses ranging from 1.5×10^{-2} to 1.5×10^{-4} mg. Fe, and the offspring were tested for sex-linked recessive lethals by the Muller-5 technique. Only 10 lethals were recovered from 5,998 treated chromosomes. Since this is within the control range, it was concluded that iron-dextran is nonmutagenic in *Drosophila*, and that, while this result does not altogether exclude the possibility of its mutagenicity in mammals, its primary and major site of cellular damage probably lies outside the nuclear genes. So far as its carcinogenicity is concerned, it is perhaps relevant that Lindegren, Nagai, and Nagai (*32*), in studies of the induction of respiratory deficiency in yeast by manganese, copper, cobalt, and nickel, point out that many effects ascribed to gene mutation may be due to damage to autonomous cytoplasmic particles, and that physical carcinogenic influences such as ultraviolet radiation and X rays have often been demonstrated to produce stable variations in the extrachromosomal apparatus.

Although clearly the question at this stage cannot be decided, equally can it by no means be excluded that the action is specifically dependent upon iron. This at once raises a host of intriguing possibilities, bearing upon the mechanisms of carcinogenic action, and the disturbances of iron metabolism in the cancer cell and tumor-bearing organism. Further, the involvement of metals in carcinogenesis, through the complexing and catalytic power of their ions, and their directive activity in many biosyntheses, may have more fundamental implications than have hitherto been suspected. We have already been reminded of the metalloid quality of the carcinogenic hydrocarbons, arising from the electron mobilities in their aromatic rings. It may also prove that chelation processes have greater importance than has been realized, as is now seen in the fields of pharmacology (*33*), chemotherapy (*34*), and plant growth substances (*35, 36*). In the last connection must be considered a general principle which has emerged over the last few years from the work of Clayson and others (*37–39*), concerning the possible importance of *o*-hydroxy derivatives as key metabolites of the carcinogenic amines. These substances could function as chelating agents, and the avidity of various *o*-aminophenols for metallic ions has recently been investigated by Sims (*40*). Although the same

would not, however, apply in the case of certain methoxy homologues which are equally carcinogenic, nevertheless the possibility must still be explored, as Boyland has suggested, of the possible role and significance of mixed chelate formation, as a stage in the action of particular carcinogens at least.

Although the situation is by no means clear or simple, certain restricted associations are already known between iron deposition and the incidence of specific types of malignant disease—as in Argyll Campbell's early description of the effects of precipitated silica and of iron oxide on the incidence of primary tumors of the lung in mice (*2*), the liability to carcinoma of the liver in hemochromatosis (*41*), and the frequency of cancer of the lung in hematite miners (*3*, *4*). Although siderosis might be suspected as contributory in the etiology of cancer of the liver in the South African Bantu, in this case there does not, however, appear to be any convincing evidence (*42–45*).

While the carcinogenicity of iron-dextran is obviously of much interest in its own right, it also recalls many well-founded observations in the literature, suggesting interference by carcinogenic agents with iron metabolism, hematopoiesis, and the hemoprotein enzymes. A few examples are:

Warren (*46*) and Parsons and Warren (*47*) studied the liberation of iron by treatment with carcinogens. Strong (*48–50*), in work which was largely supported by Goulden and Warren (*51*, *52*), described an anemia occurring in mice of mammary cancer-susceptible lines prior to tumor development, (but not in nonsusceptible pure lines), and reported a contrast in the iron content of the tissues of high- and low-cancer strains. Such examples would appear to have their counterparts in certain aspects of the anemia of cancer and the Plummer-Vinson syndrome (*53–56*). Concerning the iron content of tumor cells themselves, Rawlinson (*57*) made the observation that, on malignant transformation, the epithelial cells of the mammary gland of C3H mice appeared very abruptly to lose the property of accumulating iron. In a study of the distribution of various trace elements (zinc, molybdenum, manganese, chromium, tin, copper, nickel, aluminium, silver, lead, cobalt, and iron), as between normal and malignant human liver, Olson and others (*58*) recorded that in all the tumor tissues these elements were markedly decreased.

Next there is the striking so-called "hemolymph" change produced by many different kinds of carcinogens, almost diagnostically, which was studied by Lasnitzki and Woodhouse (*59*, *60*), Haddow and coworkers (*61*), and more recently by Elson (*62*), but whose underlying meaning still escapes us. There is also the propensity of certain carcinogens to produce methemoglobinemia, *e.g.*, various aminostilbenes (*61*) and azo-dyestuffs (*63*). Apart from such changes during the course of carcinogenesis, well-recognized and perhaps related alterations may also accompany the course of tumor growth, *e.g.*, variable reduction in the amount or activity of such hemoprotein enzyme systems as catalase (*64*) and cytochrome *c*, and an almost complete deficiency of porphyrins (*65*) (for which last it may be noted, however, tumors are markedly avid). Since not only the catalase activity but also the hemoglobin concentration is markedly lowered in tumor-bearing animals, Greenstein had suggested that this interference with catalase and hemoglobin synthesis is concerned with the formation of the hematoporphyrin nucleus. In their studies of the respiratory pigments of tumor cells, Chance and his colleagues (*66*, *67*) have found that while the concentration of cytochrome in the intact cell may

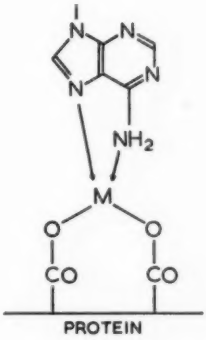
be adequate, the respiratory system is not used to the extent of its capabilities. Marked inconsistencies may be found especially affecting cytochrome *c*, a finding supported by preliminary observations carried out in the course of the present work, on our behalf, by Dr. E. F. Hartree. Yet the possibility is still not excluded, that an iron-mediated competitive or other interference with the cytochrome system could lead to the imposition of a respiratory change or mutation. Much interest has also developed (*68*) in a possible association between malignancy and the porphyrin content of tissues, the apparently frequent inability of tumor cells to synthesize the tetrapyrrole system, and the ability of neoplasms, and also perhaps rapidly growing normal tissues (*69*), to concentrate injected porphyrin. Of particular interest in the present connection is an iron-incorporating enzyme, described by Lochhead and Goldberg (*70*), transferring iron to protoporphyrin for the biosynthesis of heme. Yet another metalloflavoprotein closely involved in iron metabolism is xanthine oxidase (*71*); thus Mazur and his coworkers (*72*) have provided data suggesting that the mechanism of ferritin-iron reduction involves a transfer of electrons from the iron atoms of xanthine oxidase to those of ferritin, each iron atom in association with sulfydryl groups. Further, the physiological stimulus which results in accelerated release of iron from hepatic ferritin to the plasma appears to be tissue hypoxia (*73*, *74*). This produces an increase in xanthine oxidase substrates and a consequent reduction and release of ferritin iron to the plasma. Hence, the ferritin-xanthine oxidase system constitutes part of a homeostatic mechanism for the regulation of iron levels. The significance of these observations is obvious, in relation to the possible importance of xanthine oxidase as a limiting enzyme in certain tumors.

Apart from these observations, many others attest to the possibly key importance of iron metabolism in neoplasia—altogether omitting the views and contributions of such pioneers as Warburg and de Hevesy. So far as concerns deficiencies in iron metabolism, it is not always clear to what extent these are specific or merely secondary. In a recent study, Dorothy Ley (personal communication) has observed such defects in 80 percent of cases of advanced malignant disease with metastases, *e.g.*, inefficiency in iron-binding, and a reduced level of serum iron, together producing a marked reduction in the total iron-carrying capacity.

Lastly must be mentioned a relationship which is certainly intriguing and which may be important, namely, that between iron deposition and the aging of tissues and cells, a subject studied by Zondek and Karp (*75*) 25 years ago when they observed, at a definite middle period in life, that the iron content of epithelial organs is increased up to 200 percent; this value is maintained until death. It had also been observed by Warren and Goulden (*52*) that, in some species, aging is accompanied by an increase in the nonheme iron content of the tissues.

If the carcinogenicity of the iron-dextran preparation were indeed a function of the metal, either alone or in its complexed form, it would then be tempting to speculate as to possible mechanisms. Introduction of excess metal into what is certainly a delicately balanced system of metals and metal enzymes could undoubtedly result in maldistribution (*see* *76*) and so interfere with function, and with what Drabkin (*77*) has called "the precarious homeostasis of iron"—"the tight squeeze of iron between the evils of impoverishment and overabundance . . . ." Such interference could certainly and very readily impinge on the respiratory chain, either through competitive interference with the cytochrome system, leading to the imposition of a respiratory mutation as has already been considered, or, as Elson has suggested, through the

combination of iron with protein, resulting in an abnormal hemoglobin-like compound and competitive anoxia. It might also proceed via nucleotide metabolism, by many different routes of which one is, however, perhaps especially worthy of attention, namely, the withdrawal of diphosphopyridine nucleotide (DPN) from its functional form through the introduction of a metal with which it will readily chelate. In any such studies, much could be learned from what is already known of the physicochemical role of iron in histological staining, and especially its mordant action (*78*, *79*). According to Wigglesworth (*80*), the substrates mainly responsible for the uptake of iron are the nucleic acids and proteins. The staining capacity of different proteins runs parallel with the number of free carboxyl groups which they contain, and it is concluded that iron forms nonionizing complexes with carboxyl and phosphoric acid groups, the intensity of staining being a measure of the relative abundance of such groups in the proteins and nucleic acids present, and of the concentration density of these substances. In the staining of nuclei and chromosomes with iron, the proteins are at least as important as the nucleic acids. The iron taken up is mostly in the ferric state but is partially converted to ferrous iron by reducing substances, chiefly SH groups. It is of some related interest that Kirby (*81*) has described a possible means whereby DNA could form a complex (I) through a metal (M) with a protein containing aspartic or glutamic acid residues—in a paper suggesting the importance of metal bonding as well as ionic bonding, in the DNA structure. Incidentally, Zamenhof (*82*) has warned of the importance of the avoidance of traces of iron or rust ($Fe^{++} \rightarrow Fe^{+++}$) in the preparation of DNA, where it leads to rapid degradation.



(I)

Lastly, there must not be neglected the known effects of iron in the inactivation of vitamin E. In their studies of the changes associated with the accumulation of excessive amounts of iron in various organs of the rat, Golberg and Smith (*23*) point out that the sequelae closely resemble

many of the characteristic pathological and biochemical changes observed in rats deficient in vitamin E—for example, ceroid formation, the production of fat peroxides ("brown fat") hemolysis *in vitro*, "brown" uterus, testicular atrophy, renal autolysis, reduced liver vitamin A, and incisor depigmentation: Ceroid formation appears to be favored by such prooxidants as the iron porphyrins and other chelates, and by Fe from ferritin, and inhibited by the antioxidants vitamin E, glutathione, cysteine, and ascorbic acid.

Before concluding, it must again be stressed that the carcinogenicity of iron-dextran in the mouse and rat has been demonstrated thus far only through the use of massive doses. The body content of iron is approximately 2 mg. in the mouse and about 10 mg. in the adult rat (*83*), while a single dose of 0.2 cc. Imferon will itself introduce 10 mg. Fe. Although such overloading certainly entails systemic changes, there is no reason to believe that the carcinogenic reaction is dependent on these, or that it is other than a preponderantly local action. It may indeed mainly be determined by that fraction of the dose which is poorly absorbed or locally retained. In studies of the absorption of radioactive iron-dextran in pregnancy, Evans and Ramsey (*84*) showed that about 80 percent of a dose of Fe^{59}-dextran, given intramuscularly in the gluteal mass, was absorbed within 3 weeks after injection, but that recorded activity then diminished at only a reduced rate, over a period which might be as long as 6 months. On the basis of body weight, the "clinical dose" in the mouse would be only one one-hundredth or one one-hundred fiftieth of the dose administered in the present experiments. Tests are already under way to examine doses of this order in the mouse and rat, but are as yet incomplete. Meantime, however, Golberg and his colleagues (personal communication) have administered repeated doses to a total of 80 times the clinical dose to rats, rabbits, mice, guinea pigs, and dogs, without any apparent effect on health. In a series of 414 stock mice receiving repeated injections by various routes up to a total of 170 times the clinical dose during a period of 88 weeks, 9 tumors were observed, of which 8 were regarded as spontaneous and only 1 was a spindle-cell sarcoma at the injection site. In a series of 527 albino and hooded rats receiving up to a total of 210 times the clinical dose by repeated intramuscular injection, 11 sarcomas appeared at the injection site after 82 to 118 weeks. In 5,200 stock mice receiving up to 170 times the clinical dose, no tumors were observed up to 48 weeks, though the experiment continues. While Golberg has therefore obtained a small yield of tumors attributable to iron-dextran, in certain of his experiments, this in no way compares with the much greater incidence reported in the present paper, and by Richmond. In considering questions of hazard, it would appear most probable that the carcinogenic response is decided primarily by the absolute amount administered, and only secondarily, if at all, by the relation of this to the total-body weight. Even if quite negative results were to be obtained in mice, with doses that bear the same relation to those used in man, as do the body weights of mice and men, this would not necessarily

in any way diminish a hazard attaching to the absolute amounts that are in fact administered to humans.

In conclusion, we are still ignorant of the real significance of the carcinogenic action of metals, whether this is particular or relatively nonspecific, or whether it possesses wider and more fundamental meaning. So far as the iron-dextran complex is concerned, it has already been emphasized that we know no more than that it is carcinogenic for the mouse and rat. The specificity of, or necessity for, the iron component is still quite undecided, but in view of the unique functions of iron the observation could clearly be of profounder significance. In any event it has been thought not only desirable but necessary to draw attention to Richmond's recent and important discovery, and to confirm and extend it.

Note Added in Proof, December 12, 1959

Since this paper was submitted, additional tumors (all at the injection site) have been observed as follows: 1 rat sarcoma in the Imferon series, 1 sarcoma in the series of Chinese hamsters receiving 0.1 cc. Imferon weekly, and 1 sarcoma each in the series of mice treated with Imferon batches 145Z and 107/59 (*see* page 114). It is of much interest that 2 further sarcomas have appeared in the mice treated with aluminium-dextran batch 6438/7 (*see* table 1) and no fewer than 5 in a similar series (batch 6463/2) not referred to above. Lastly, in experiments to test successively lower dosages, 1 sarcoma has appeared after 8 months, in a series of mice receiving 0.05 cc. Imferon weekly by subcutaneous injection. These experiments continue.

REFERENCES

(*1*) Hueper, W. C.: Experimental and histological studies of metal cancers of the lung. (Abstract.) *In* Seventh Internat. Cancer Congress, London, 1958, pp. 74–75.

(*2*) Campbell, J. A.: Effects of precipitated silica and of iron oxide on the incidence of primary lung tumours in mice. Brit. M. J. 2: 275–280, 1940.

(*3*) Faulds, J. S., and Stewart, M. J.: Carcinoma of the lung in haematite miners. J. Path. & Bact. 72: 353–366, 1956.

(*4*) Bonser, G. M., Faulds, J. S., and Stewart, M. J.: Occupational cancer of the urinary bladder in dyestuffs operatives and of the lung in asbestos textile workers and iron-ore miners. Am. J. Clin. Path. 25: 126–134, 1955.

(*5*) Richmond, H. G.: Induction of sarcoma in rats by an iron-dextran complex. Scottish M. J. 2: 169, 1957.

(*6*) ———: Induction of sarcoma in the rat by iron-dextran complex. Brit. M. J. 1: 947–949, 1959.

(*7*) ———: The carcinogenicity of an iron-dextran complex. *In* Cancer Progress, I. London, Butterworth & Co., 1959. In press.

(*8*) Haddow, A.: The possible role of metals and of metal chelation in the carcinogenic process. *In* Ciba Foundation Symposium on Carcinogenesis: mechanisms of action. London, Churchill, 1959, pp. 300–306.

(9) GILLMAN, J., and GILLMAN, T.: The pathogenesis of experimentally produced lymphomata in rats (including Hodgkin's-like sarcoma). Cancer 5: 792–846, 1952.

(10) MARTIN, L. E., BATES, C. M., BERESFORD, C. R., DONALDSON, J. D., MCDONALD, F. F., DUNLOP, D., SHEARD, P., LONDON, E., and TWIGG, G. D.: The pharmacology of an iron-dextran intramuscular haematinic. Brit. J. Pharmacol. 10: 375–382, 1955.

(11) CAPPELL, D. F., HUTCHINSON, H. E., HENDRY, E. B., and CONWAY, H.: A new carbohydrate-iron haematinic for intramuscular use. Brit. M. J. 2: 1255–1257, 1954.

(12) MCDONALD, F. F., DUNLOP, D., and BATES, C. M.: An effective treatment for anaemia of piglets. Brit. Vet. J. 111: 403–407, 1955.

(13) BROWNLIE, W. M.: The treatment of piglet anaemia. Vet. Rec. 67: 350–354, 1955.

(14) BARBER, R. S., BRAUDE, R., and MITCHELL, K. G.: Studies on anaemia in pigs. I. The provision of iron by intramuscular injection. Vet. Rec. 67: 348–349, 1955.

(15) KERNKAMP, H. C. H.: A parenteral hematinic, 'Imferon' for the control of iron-deficiency anemia in baby pigs. N. Amer. Vet. 38: 6–9, 1957.

(16) Intramuscular iron therapy. Brit. M. J. 2: 1281, 1954.

(17) NISSIM, J. A.: Deposition of iron in the testes after administration of an iron-dextran complex. Lancet 1: 701–702, 1955.

(18) ———: Evidence of a spermatocytotrophic hormone produced by the interstitial cells of the testis. J. Physiol. 131: 27P–28P, 1956.

(19) ———: The nature and chemical affinities of the interstitial cells of the testis. (Abstract.) J. Endocrinol. 13: 36–37, 1956.

(20) GOLBERG, L., SMITH, J. P., and MARTIN, L. E.: Effects of massive iron overload in the rat. Nature, London 179: 734, 1957.

(21) ———: The effects of intensive and prolonged administration of iron parenterally in animals. Brit. J. Exper. Path. 38: 297–311, 1957.

(22) BERESFORD, C. R., GOLBERG, L., and SMITH, J. P.: Local effects and mechanism of absorption of iron preparations administered intramuscularly. Brit. J. Pharmacol. 12: 107–114, 1957.

(23) GOLBERG, L., and SMITH, J. P.: Changes associated with the accumulation of excessive amounts of iron in certain organs of the rat. Brit. J. Exper. Path. 39: 59–73, 1958.

(24) NORDÉN, Å.: Studies of iron-59 labeled iron-dextran complex following intramuscular injection. Nord. med. 58: 1216–1221, 1957.

(25) KARLEFORS, T., and NORDÉN, Å.: Studies on iron-dextran complex. Acta med. scandinav. Supp. 342, 1958, 54 pp.

(26) TURNER-WARWICK, R. T.: Intravital techniques for the demonstration of lymphatics. Ann. Roy. Coll. Surgeons England 24: 101–109, 1959

(27) MACKENZIE, A., and TINDLE, J.: Determination of plasma-volume using intravenous iron dextran. Lancet 1: 333–335, 1959.

(28) RICHTER, G. W.: The cellular transformation of injected colloidal iron complexes into ferritin and hemosiderin in experimental animals. A study with the aid of electron microscopy. J. Exper. Med. 109: 197–216, 1959.

(29) HUEPER, W. C.: Experimental carcinogenic studies in macromolecular chemicals. I. Neoplastic reactions in rats and mice after parenteral introduction of polyvinyl pyrrolidones. Cancer 10: 8–18, 1957.

(30) ———: Carcinogenic studies on water-soluble and insoluble macromolecules. A.M.A. Arch. Path. 67: 589–617, 1959.

(31) LUSKY, L. M., and NELSON, A. A.: Fibrosarcomas induced by multiple subcutaneous injections of carboxymethylcellulose (CMC), polyvinylpyrrolidone (PVP), and polyoxyethylene sorbitan monostearate (Tween 60). (Abstract.) Fed. Proc. 16: 318, 1957.

(32) LINDEGREN, C. C., NAGAI, S., and NAGAI, H.: Induction of respiratory deficiency in yeast by manganese, copper, cobalt, and nickel. Nature, London 182: 446–448, 1958.

(33) CHENOWETH, M. B.: Chelation as a mechanism of pharmacological action. Pharmacol. Rev. 8: 57–87, 1956.

(34) ALBERT, A.: Metal-binding agents in chemotherapy: the activation of metals by chelation. *In* The Strategy of Chemotherapy. Society for General Microbiology Symposium, 8th. London, Cambridge Univ. Press, 1958, pp. 112–138.

(35) HEATH, O. V. S., and CLARK, J. E.: Chelating agents as growth substances. Nature, London 178: 600–601, 1956.

(36) ———: Chelating agents as plant growth substances. A possible clue to the mode of action of auxin. Nature, London 177: 1118–1121, 1956.

(37) CLAYSON, D. B.: A working hypothesis for the mode of carcinogenesis of aromatic amines. Brit. J. Cancer 7: 460–471, 1953.

(38) BRADSHAW, L., and CLAYSON, D. B.: Metabolism of two aromatic amines in the dog. Nature, London 176: 974–975, 1955.

(39) CLAYSON, D. B., JULL, J. W., and BONSER, G. M.: The testing of orthohydroxyamines and related compounds by bladder implantation and a discussion of their structural requirements for carcinogenic activity. Brit. J. Cancer 12: 222–230, 1958.

(40) SIMS, P.: The stability constants of some metal chelates of *ortho*-aminophenols. J. Chem. Soc. 3648–3649, 1959.

(41) WILLIS, R. A.: Haemochromatosis, with special reference to supervening carcinoma of liver. M. J. Australia 2: 666–669, 1941.

(42) HIGGINSON, J., GERRITSEN, T., and WALKER, A. R. P.: Siderosis in the Bantu of Southern Africa. Am. J. Path. 29: 779–815, 1953.

(43) HIGGINSON, J.: Pathogenesis of liver cancer in the Johannesburg area. (S. Africa). Part I. Acta Unio internat. contra cancrum 13: 590–598, 1957.

(44) BUTT, E. M., and HIGGINSON, J.: Trace element pattern in liver disease and liver carcinoma. Part II. Acta Unio internat. contra cancrum 13: 599–601, 1957.

(45) HIGGINSON, J., and OETTLE, A. G.: The incidence of liver cancer in South Africa. Part III. Acta Unio internat. contra cancrum 13: 602–605, 1957.

(46) WARREN, F. L.: Estimations of iron in the lymph glands of mice during treatment with a carcinogenic compound. Biochem. J. 33: 729–733, 1939.

(47) PARSONS, L. D., and WARREN, F. L.: Cellular changes in the spleen and lymph glands in mice used for carcinogenic and related experiments, with special reference to giant cells of spleen. J. Path. & Bact. 52: 305–321, 1941.

(48) STRONG, L. C.: Hemoglobin levels in various degrees of susceptibility to spontaneous tumors. Am. J. Cancer 27: 500–509, 1936.

(49) FRANCIS, L. D., and STRONG, L. C.: Hemoglobin studies on blood of female mice of CBA strain: effects of age, diet, strain, and reproduction. Am. J. Physiol. 124: 511–516, 1938.

(50) STRONG, L. C., and FRANCIS, L. D.: Differences in hemoglobin values in the blood of breeder female mice; a comparison between cancer-susceptible and cancer-resistant strains. Am. J. Cancer 38: 399–403, 1940.

(51) GOULDEN, F., and WARREN, F. L.: The hemoglobin content of the blood of mice of the RIII and CBA strains. Cancer Res. 4: 421–424, 1944.

(52) WARREN, F. L., and GOULDEN, F.: The non-heme iron content of the tissues of mice of high-cancer and low-cancer strains. Cancer Res. 4: 417–420, 1944.

(53) PRICE, V. E., and GREENFIELD. R. E.: Anemia in cancer. Advances Cancer Res. 5: 199–290, 1958.

(54) GREENFIELD, R. E., GODFREY, J. E., and PRICE, V. E.: Studies on the anemia of tumor-bearing animals. I. Distribution of radioiron following the injection of labeled erythrocytes. J. Nat. Cancer Inst. 21: 641–656, 1958.

(55) GELLHORN, A., HYMAN, G., and ULTMANN, J.: The mechanism of the anemia of cancer. New York Med. 13: 614–626, 1957.

(56) VON HEVESY, G.: Die Krebsanämie. Naturwiss. Rdsch. 11: 247–252, 1958.

(57) RAWLINSON, H. E.: The iron content of the resting mammary glands of normal and tumour-bearing C3H mice. Acta Unio internat. contra cancrum 12: 711–717, 1956.

(58) OLSON, K. B., HEGGEN, G., EDWARDS, C. F., and GORHAM, L. W.: Trace element content of cancerous and noncancerous human liver tissue. Science 119: 772–773, 1954.

(59) LASNITZKI, A., and WOODHOUSE, D. L.: Formation of haemolymph nodes in rats treated with 1:2:5:6-dibenzanthracene. Nature, London 150: 660, 1942.

(60) ———: Effect of 1:2:5:6-dibenzanthracene on lymph-nodes of rat. J. Anat. 78: 121–129, 1944.

(61) HADDOW, A., HARRIS, R. J. C., KON, G. A. R., and ROE, E. M. F.: The growth-inhibitory and carcinogenic properties of 4-aminostilbene and derivatives. Phil. Trans. A. Roy. Soc. London 241: 147–195, 1948.

(62) ELSON, L. A.: Hematological effects of the alkylating agents. Ann. New York Acad. Sc. 68: 826–833, 1958.

(63) NEISH, W. J. P.: Effect of size and age of female rats on their response to the methaemoglobinogenic action of 3′-methyl-4-dimethylaminoazobenzene. Nature, London 178: 1350–1351, 1956.

(64) GREENSTEIN, J. P.: Further studies of the liver catalase activity of tumor-bearing animals. J. Nat. Cancer Inst. 3: 397–404, 1943.

(65) FIGGE, F. H. J.: The relationship of pyrrol compounds to carcinogenesis. *In* A.A.A.S. Research Conf. Cancer (1944), pp. 117–128, 1945.

(66) CHANCE, B., and CASTOR, L. N.: Some patterns of the respiratory pigments of ascites tumors of mice. Science 116: 200–202, 1952.

(67) CHANCE, B., and HESS, B.: Spectroscopic evidence of metabolic control. Science 129: 700–708, 1959.

(68) RIMINGTON, C.: Haem pigments and porphyrins. Ann. Rev. Biochem. 26: 561–586, 1957.

(69) KENNEDY, G. Y.: The chemistry and metabolism of some porphyrins and porphyrin derivatives. Univ. Sheffield, Ph.D. Thesis, 1954.

(70) LOCHHEAD, A. C., and GOLDBERG, R.: Transfer of iron to protoporphyrin for haem biosynthesis: role of ascorbic acid and glutathione. Lancet 2: 271–272, 1959.

(71) BERGEL, F., and BRAY, R. C.: The role of metals in oxidations catalysed by xanthine oxidase and by other metalloflavoproteins. Symp. Biochem. Soc. 15: 64–75, 1958.

(72) MAZUR, A., and GREEN, S.: Relation of iron to sulphydryl groups in ferritin. *In* Sulphur in Proteins. (Benesch, R., *et al.*, ed.) New York, Academic Press, Inc., 1959, pp. 189–196.

(73) MAZUR, A., GREEN, S., SAHA, A., and CARLETON, A.: Mechanism of release of ferritin iron *in vivo* by xanthine oxidase. J. Clin. Invest. 37: 1809–1817, 1958.

(74) GREEN, S., SAHA, A. K., CARLETON, A. W., and MAZUR, A.: Release of storage iron to the plasma by xanthine oxidase after purine administration. (Abstract.) Fed. Proc. 17: 233, 1958.

(75) ZONDEK, S. G., and KARP, J.: The relationship of iron with the ageing of cells. Biochem. J. 28: 587–591, 1934.

(76) GOLDBLATT, M. W., and GOLDBLATT, J.: Some Aspects of Industrial Toxicology. *In* Industrial Medicine and Hygiene. (Merewether, E. R. A., ed.). London, Butterworth & Co., Ltd., 1956, vol. 3, p. 473.

(77) DRABKIN, D. L.: Metabolism of the hemin chromoproteins. Physiol. Rev. 31: 345–431, 1951.

(78) BAKER, J. R.: Principles of Biological Microtechnique. London, Methuen & Co., Ltd., 1958.

(79) VICKERSTAFF, T.: Physical Chemistry of Dyeing. 2d ed., Edinburgh, Oliver & Boyd, Ltd., 1954.

(80) WIGGLESWORTH, V. B.: The role of iron in histological staining. Quart. J. Micr. Sc. 93: 105–118, 1952.

(81) KIRBY, K. S.: A new method for the isolation of deoxyribonucleic acids: Evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J. 66: 495–504, 1957.

(82) ZAMENHOF, S.: Deoxyribonucleic acid (DNA). Biochem. Prep. 6: 8–12, 1958.

(83) UNDERWOOD, E. J.: Trace Elements in Human and Animal Nutrition. New York, Academic Press, Inc., 1956.

(84) EVANS, L. A. J., and RAMSEY, N. W.: Absorption studies on radioactive iron-dextran in pregnancy. Lancet 2: 1192–1196, 1957.

PLATES

PLATE 2

FIGURE 1.—Electron micrograph (tungsten-shadowed) of iron-dextran particles and clusters. × 160,000

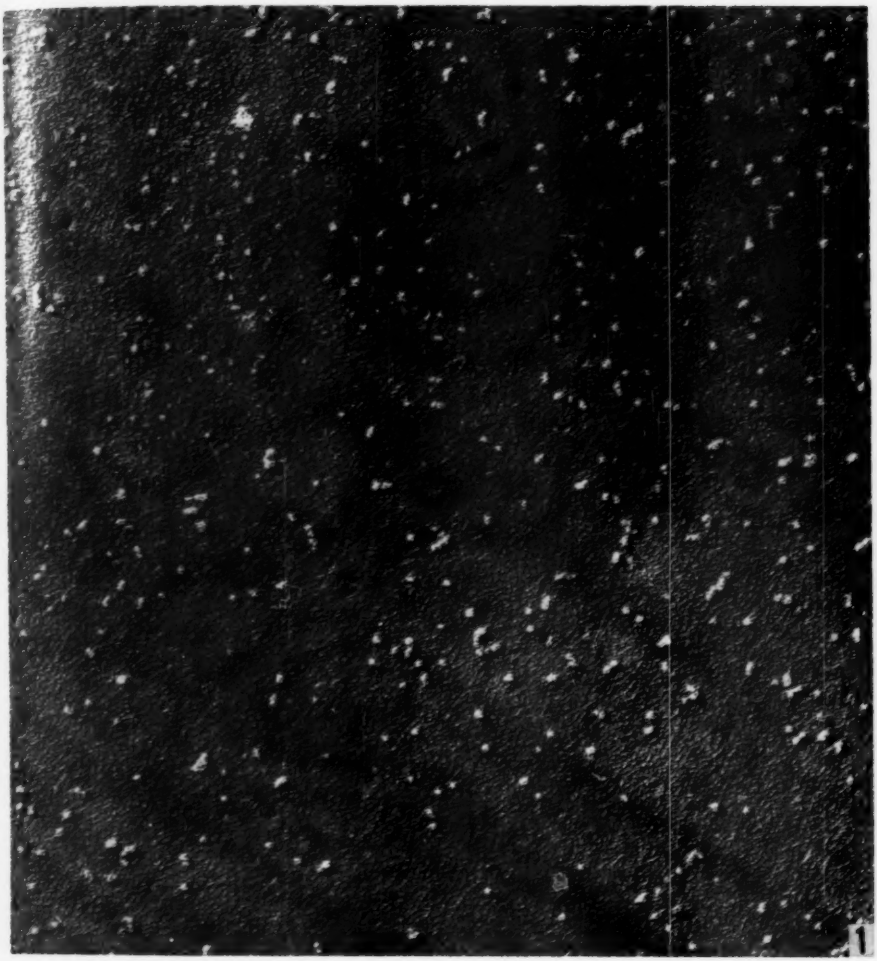


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PLATE 3

FIGURE 2.—Epilation as a result of repeated subcutaneous injection of iron-dextran.

FIGURE 3.—Spindle-cell sarcoma in the mouse, induced by iron-dextran 0.2 cc. subcutaneously on 13 occasions. × 480

FIGURES 4 AND 5.—Examples of iron-dextran-induced sarcomas in the first series in the mouse. × 320

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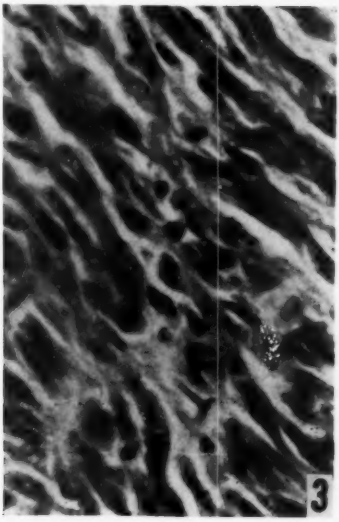
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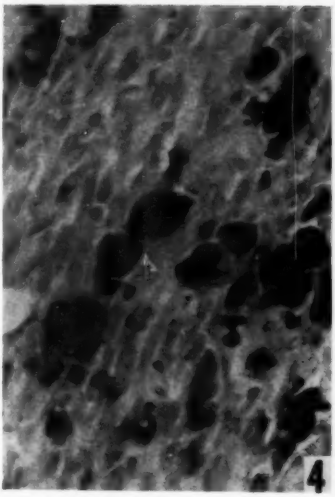
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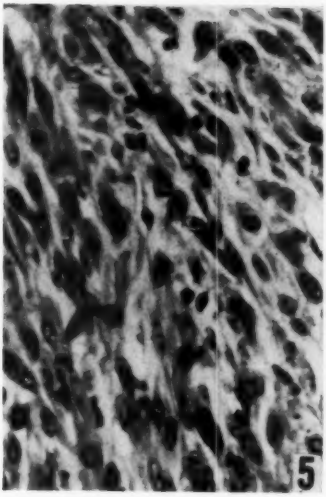
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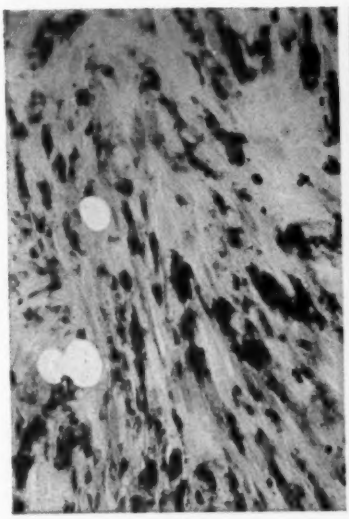
PLATE 4

FIGURE 6.—The Prussian-blue reaction in same tumor shown in figure 3. × 240

FIGURE 7.—Example of iron-dextran-induced sarcoma in the first series in the mouse. × 320

FIGURE 8.—Coincident hypertrophic areas in liver. × 60

FIGURE 9.—Seventh primary iron-dextran-induced sarcoma in the second mouse series. × 158



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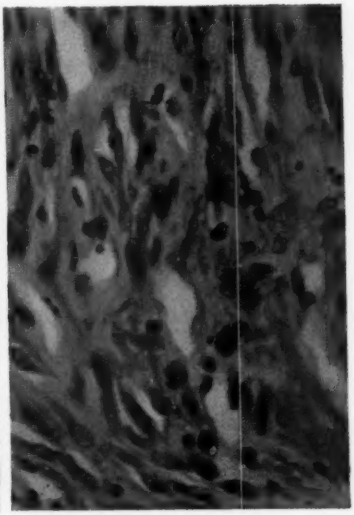

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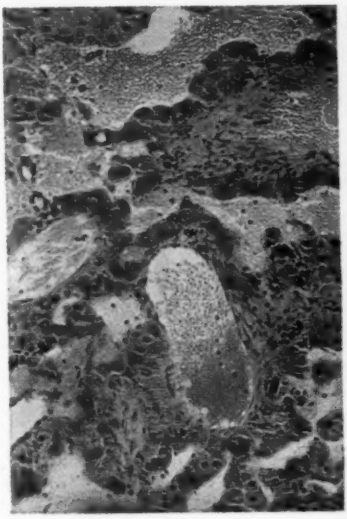

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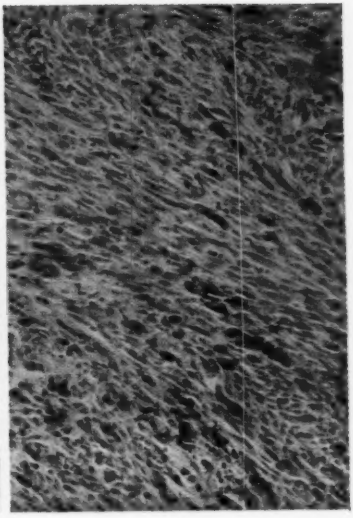

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PLATE 5

FIGURE 10.—Prussian-blue reaction in iron-dextran-induced sarcoma. × 200

FIGURE 11.—Primary iron-dextran-induced mouse sarcoma. × 240

FIGURE 12.—Primary induced sarcoma with tumor mass in hind limb.

FIGURE 13.—Coincident bronchogenic carcinoma.

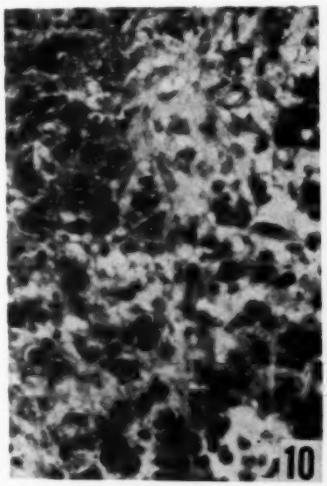

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PLATE 6

FIGURE 14.—Primary iron-dextran-induced mouse sarcoma: islets of ferritin granules in cytoplasm.

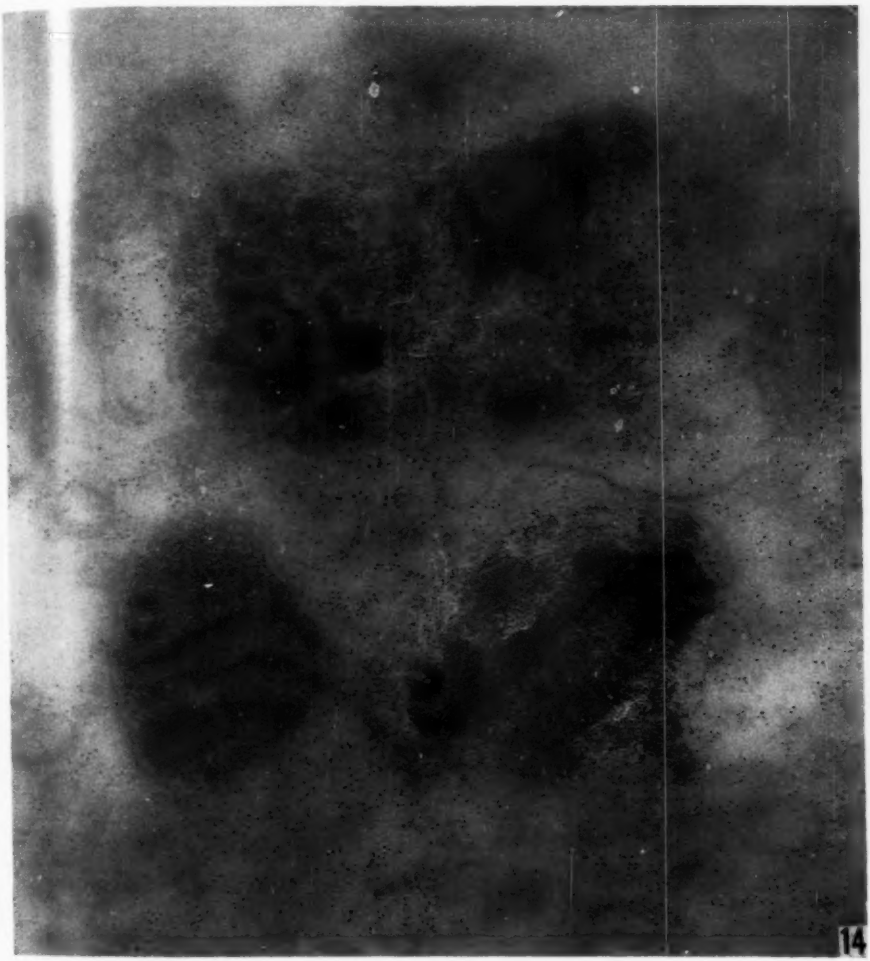
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PLATE 7

FIGURE 15.—Ferritin granules in splenic macrophage (18-month-old rat). × 160,000

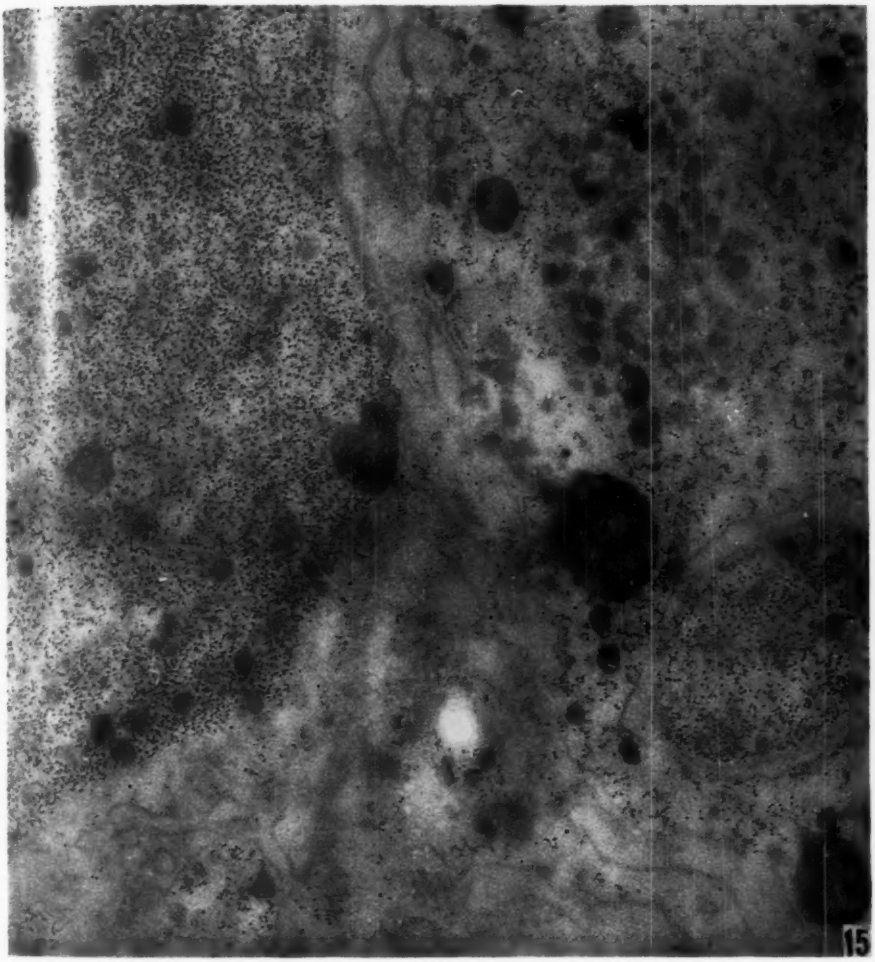
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PLATE 8

FIGURES 16 AND 17.—Epithelial downgrowth overlying primary induced sarcomas. (Fig. 16 × 158; fig. 17 × 80.)

FIGURES 18 AND 19.—Epithelioma induced at site of injection of a poorly absorbed specimen of iron-dextran (fig. 19 × 90).

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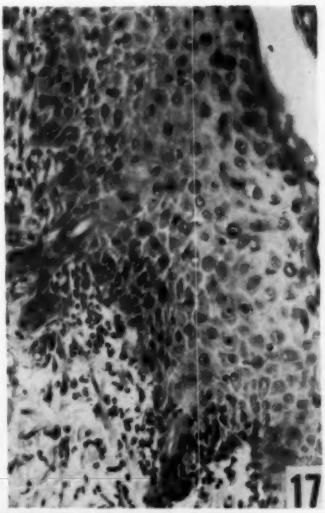
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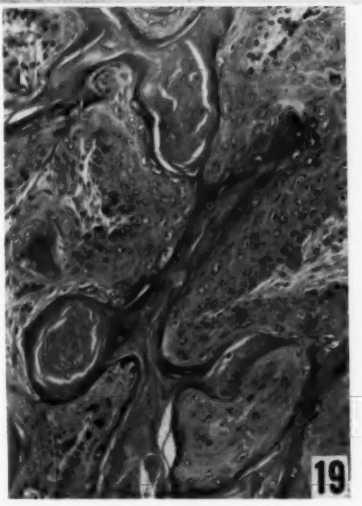
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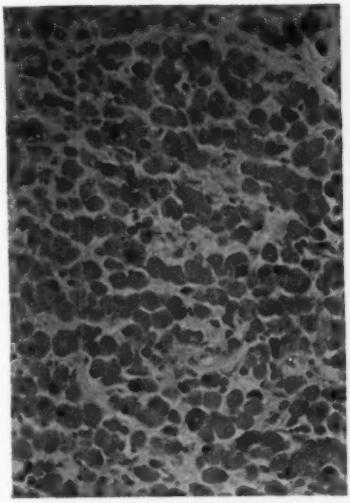
PLATE 9

FIGURE 20.—Induced histiocytoma of mouse. × 158

FIGURE 21.—Spherical intranuclear iron-containing inclusion bodies in hepatic parenchyma. × 480

FIGURE 22.—Benign iron-dextran-induced rat histiocytoma. × 260

FIGURE 23.—The distribution of iron in intestinal mucosa. × 160



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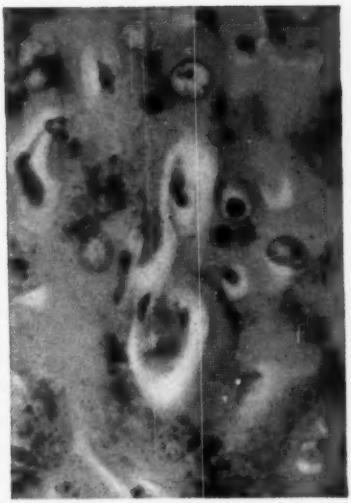

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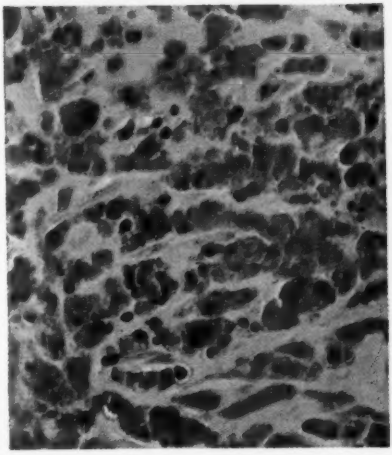

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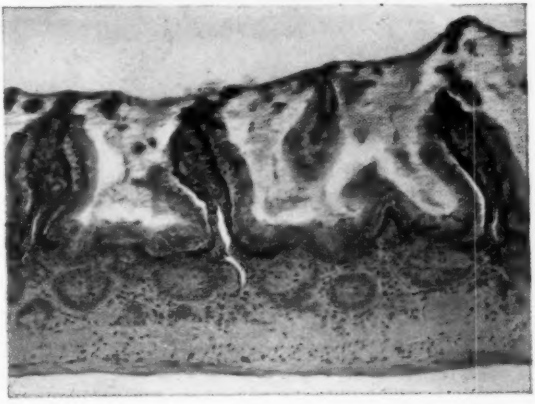

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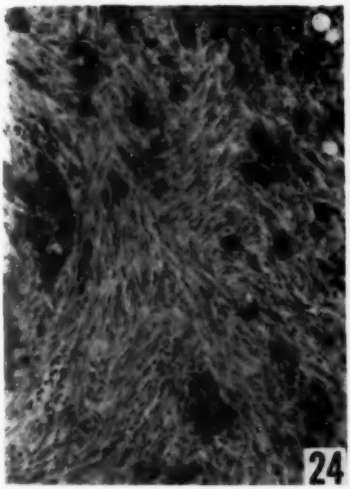
PLATE 10

FIGURE 24.—Primary fibrosarcoma induced by repeated subcutaneous injection of a poorly absorbed specimen of iron-dextran. This tumor had invaded the abdominal wall, with widespread lymphatic metastasis. × 160

FIGURE 25.—Intra-abdominal lymphosarcoma.

FIGURE 26.—Inhibition by iron-dextran of regrowth of hair, with tuft stimulated by injection trauma.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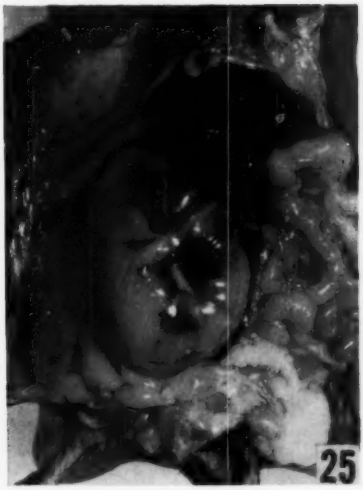
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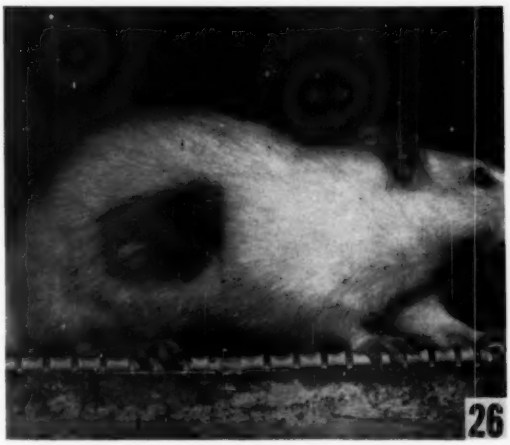
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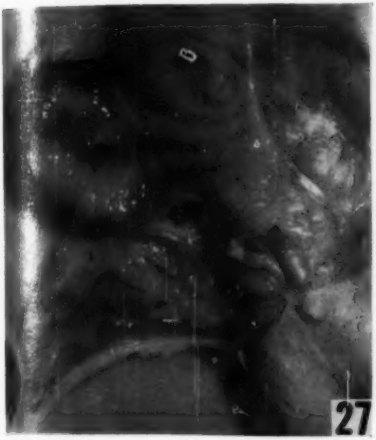
PLATE 11

FIGURE 27.—Adenocarcinoma of mammary gland in a male rat forced-fed with oral iron-dextran.

FIGURE 28.—Iron-dextran-induced hypertrophy of liver in male Chinese hamster.

FIGURE 29.—Iron-dextran-induced spindle-cell sarcoma in male rat, with dispersed pigment-laden macrophages. × 300

FIGURE 30.—Pleomorphic mouse sarcoma induced by 8-hydroxyquinoline copper complex. × 216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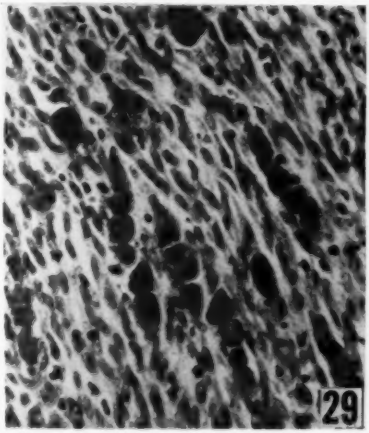
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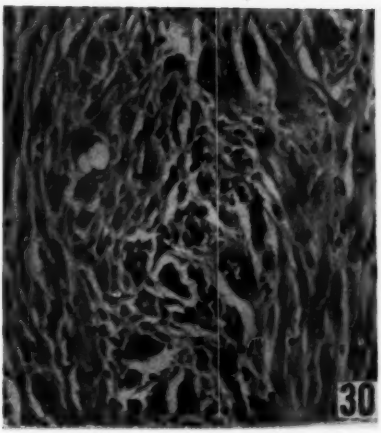
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Studies of Carcinogenicity in the Rate of Derivatives of Aromatic Amines Related to *N*-2-fluorenylacetamide [1]

HAROLD P. MORRIS,[2] CLARENCE A. VELAT,[3] BILLIE P. WAGNER,[2] MURIEL DAHLGARD,[4] *and* FRANCIS E. RAY,[4, 5, 6] *National Cancer Institute, Bethesda, Maryland, and University of Florida, Gainesville, Florida*

SUMMARY

The carcinogenic activities have been investigated in a series of 18 compounds, derivatives, and isomers of the carcinogenic *N*-2-fluorenylacetamide, and 2 acetylated azo dyes. The chemicals were incorporated into diets by dissolving them in either propylene glycol, neutral fat, or acetone. The acetone solution was first mixed with a diet constituent and the solvent was evaporated before incorporation into the diet mixture; the levels of the chemicals in the diet varied from 0.025 to 0.05 percent. The diets containing the various chemicals were fed to female Buffalo-strain rats for periods varying from an average of 3.6 to 18.0 months. The average daily intake of the chemicals varied from 2.0 to 5.1 mg. and the average total intake per rat per experiment varied from 538 to 2,693 mg. Many of the animals were observed for several months after cessation of drug feeding. All the compounds studied except the noncarcinogenic compound, Pavatrine, a commercial product, were synthesized or purified specifically for these tests. Fluorene itself was slightly carcinogenic. When diets containing this chemical were fed, for an average of 7 to 19 months, respectively, in 2 separate experiments, a few tumors appeared at several sites. *N*-(7-iodo-2-fluorenyl)acetamide was noncarcinogenic. *N*-(3-iodo-2-fluorenyl)acetamide induced 2 leukemias, a large number of ear-duct tumors, a few lung tumors, and 1 mammary-gland tumor. A multiple hepatoma and a hemangioma of the liver, in 2 of 7 rats, occurred after ingestion of *N*-(7-chloro-2-fluorenyl)acetamide for 3 months. *N*-(2-fluorenyl)2,2,2-trifluoroacetamide was a highly active carcinogen for the liver, mammary gland, ear duct, and several other sites. Hepatoma was noted in the liver of 1 rat after ingestion of *N*-(2-fluorenyl)2-fluoroacetamide for a period of 6 months. The sulfur-containing 2-acetamido-4(2′-fluoryl)thiazole was noncarcinogenic. Five tumors of various sites including 1 mammary-gland tumor were observed, after 19 months, in rats that ingested the sulfur-containing 4,4′-sulfonylbisacetanilide for 10 months. One uterine tumor was noted in a group of rats fed a diet containing *N*-2-fluorenyl-*p*-toluenesulfonamide for approximately 6 months. Of 17 rats ingesting *N*-2-fluorenylsuccinamic acid, for an average of 17 months, 6 developed hepa-

[1] Received for publication July 28, 1959.

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[5] Supported in part by research grants C-341 and C-1066 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[6] Our sincere appreciation to Dr. B. K. Weisburger and Dr. J. H. Weisburger for the synthesized compounds prepared especially for these studies.

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tomas. A few tumors of several other sites were also observed in this group. In 18 rats ingesting *N*-(9-oxo-2-fluorenyl)acetamide, there were a large number of mammary-gland tumors, 1 hepatoma, 4 ear-duct tumors, and several tumors of other types. Rats that ingested *N*-(7-methoxy-2-fluorenyl)acetamide for a year developed mammary-gland and ear-duct tumors, as well as a few tumors of several other sites. Rats fed diets containing *m*-tolylazoacetanilide or *p*-phenylazoacetanilide for a year developed no liver tumors, but a few tumors of other types were observed. Eighteen control animals, on the basal diet, which were autopsied at an average age of 18 months, developed 1 uterine adenocarcinoma, 1 pituitary adenoma, an inguinal region fibroma, and a polypoid hemangioma of the uterus. Some groups of rats ingesting the most active carcinogens lost weight during the first 2 weeks of the test period, while some other groups ingesting active carcinogens did not. Some groups ingesting compounds that were almost completely inactive as carcinogens also lost weight during the first 2 weeks of the test period. All groups except one rapidly regained this lost weight and continued to gain at a rate almost parallel to that of the control animals, for the remainder of the test periods. Thus, changes in body weight in this series of tests were not a reliable criterion of carcinogenic activity.—J. Nat. Cancer Inst. 24: 149–180, 1960.

THE CARCINOGEN *N*-2-fluorenylacetamide [7] (2-FAA) offers a unique opportunity to study the relationship of chemical structure to the development of neoplasms in the rat because it induces tumors in many different tissues. Modification of the chemical structure may drastically change the action of the chemical toward one or more sites, by enhancing or inhibiting the carcinogenic action toward that site, or by causing it to act on an entirely new site. The comparative ease of synthesizing various isomers, closely related derivatives, or structural analogues is also a distinct advantage. A preliminary report, primarily devoted to the effect of chemical alteration of the 2-FAA molecule on hepatocarcinogenesis (*1*), included brief accounts of some of the tumors produced in other tissues by chemical compounds that have been examined for carcinogenic potency in this laboratory during the last several years.

One of the purposes of these investigations has been to discover chemicals whose carcinogenic activity would permit a more precise evaluation of whether carcinogenic mechanism(s) is related to structure, metabolic activity, or biochemical action on susceptible tissue. It has already been observed that the diacetylation of 2-fluorenamine markedly changes its carcinogenic action compared to that of the mono-acetylated compound, 2-FAA (*2*, *3*). By this slight modification of the side chain a greatly enhanced carcinogenic activity of the compound toward the liver was obtained, which differed by sex and was further modified by sex hormones (*3*). Liver tumors developed rapidly in male rats, but this response was almost completely inhibited in the female, or in the castrated male receiving in addition to the carcinogen the estrogenic substance diethylstilbestrol.

N-Methylation, so important in the carcinogenic action of *N*-dimethyl-

[7] *Chemical Abstracts'* nomenclature used. Alternate names are: 2-acetylaminofluorene and 2-acetamidofluorene.

p-phenylazoaniline (DAB) (*4*) on rat liver, has a relatively slight effect on 2-FAA liver carcinogenesis, as shown by a high incidence of liver tumors after ingestion of mono- or dimethyl-fluorenylacetamide, even though extensive demethylation takes place (*5*, *6*). The elimination of the methylene bridge of 2-FAA and 2-FA to form *N*-4-biphenylacetamide or *N*-4-biphenylamine, respectively, decreased carcinogenicity of the liver (*1*, *7–11*). This decrease in liver carcinogenesis in the absence of the methylene bridge can be overcome by the substitution of the halogen fluorine in the 4′ position, as illustrated by the carcinogenic action of 4′-fluoro-4-biphenylacetamide (*7–10*). The latter compound, in addition to being a potent liver carcinogen, also induced a high incidence of epithelial neoplasms at a new site—the renal tubule. These successful efforts to alter carcinogenesis serve to highlight some of the possibilities which such an approach opens to the study of the mechanism(s) of carcinogenesis.

This report gives detailed information on a series of compounds related to fluorenamine or to certain azo dyes whose carcinogenic potency has been tested in this laboratory. These compounds may be divided into a number of 2-FAA structural types as follows: (*a*) absence of the nitrogen attached to the ring, (*b*) halogenation on the ring, (*c*) halogenation on the side chain, (*d*) positional isomers of 2-FAA, (*e*) oxidation or other changes at certain positions, (*f*) addition of sulfur-containing substituents, (*g*) lengthening of the side chain, (*h*) diacetylation, and (*i*) acetylation of two phenylazoanilides whose methyl derivatives are well known liver-specific carcinogens.

METHODS

Rats of the Buffalo strain (*12*) were used throughout this series of carcinogenic studies. Females were used almost exclusively because of their susceptibility to the induction of mammary-gland tumors. It is recognized that female rats may not be as susceptible as males to liver-tumor induction, but studies on males seldom reveal mammary-gland-cancer-inducing properties of a chemical. Often, too, there was not enough of the compound available to make adequate carcinogenic assays on both sexes. It was then necessary to choose between inadequate tests on both sexes or a more complete test on a single sex; the latter was arbitrarily selected in most cases. Tests for carcinogenicity are time-consuming and expensive so that usually only a single preliminary test of carcinogenicity was made for most of the compounds studied and described in this report.

Two basal diets were used—diet 143 (*2*), in the early experiments. This diet, composed of purified materials, was low in protein and fat. The second diet, 222 (*9*), was composed of a mixture of natural foodstuffs. It was slightly modified by replacing the 3 percent propylene glycol with an equal percentage of corn oil. The modified version was designated 222A. The change was made because of the report (*13*) that a propylene

glycol solution of 2-FAA, when injected subcutaneously into the rat, tended to increase the induction of leukemia. This observation has not been substantiated in our laboratory (*14*), after oral administration of propylene glycol containing 2-FAA.

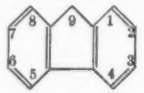
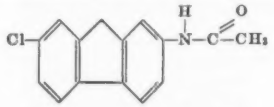
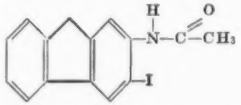
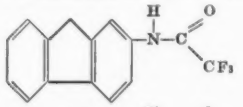
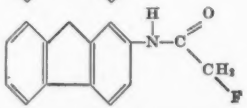
Throughout the experimental period, the rats were housed individually in raised cages with wire-cloth bottoms. The animal room was maintained at 79° ± 1° F., a desirable temperature for rats thus housed for long periods, on diets containing toxic materials. Food and water were available *ad libitum*. Body weight and food intake were obtained weekly for each animal. The diets were prepared at frequent intervals, and the excess was stored in a refrigerator until needed. Animals were checked daily, except Saturday and Sunday, for evidence of illness, and if it appeared doubtful that an animal would survive until the next check day, it was killed. Animals were sometimes found dead in spite of this checking procedure. Nevertheless, information was obtained on later microscopic examination of their tissues, which usually were not too autolyzed. Gross tumors were recorded in such cases and occasionally indicated in the results. All rats were autopsied, and the organs and tissues fixed in Zenker-formol solution, sectioned, stained with hematoxylin and eosin, and examined microscopically. Our intention was to maintain the rats on the drug-containing diet for approximately 12 months, but this depended on several factors—the quantity of the compound available for testing, its toxicity, and its carcinogenic activity. A highly active compound frequently induced tumors, early in the experiment, and as death of the rats became imminent because of large tumors, it was necessary to kill some animals while they were still on the drug. For slow-acting carcinogens, the animals could be maintained on the drug for the full 12-month period, but some compounds were so toxic that the rats often succumbed early in the experimental period. Those animals that survived 12 months on the drug were then placed on the basal control diet and observed; some survived 6 months or more. Animals 1-month old at the start of the experiment would then be 19 months of age at the termination of the experiments. The older the starting age, the older the animal when killed. The incidence of spontaneous tumors increases rather sharply in Buffalo-strain control rats after approximately 22 months of age and in those with pituitary and adrenal tumors at an even earlier age. One compound was fed continuously for 18 months, or for the entire experimental period, and a few others were fed beyond 12 months.

The chemicals used and references to their method of preparation are presented in table 1 (*15–30*).

RESULTS

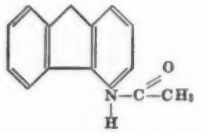
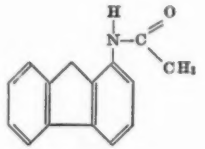
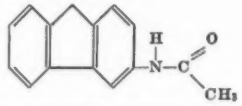
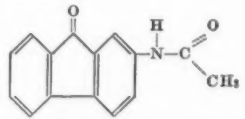
Of the control animals, one group was started on the diet at 1.8 and the other at 3.5 months of age. All controls were autopsied before the animals attained 20 months of age (table 2). The animals started at the younger age were also autopsied, on the average, about 1.5 months

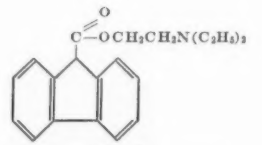
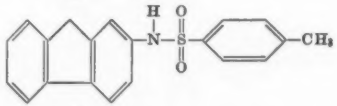
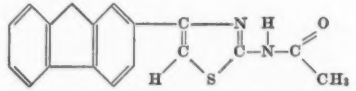
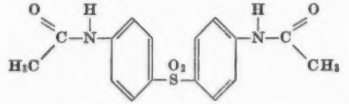
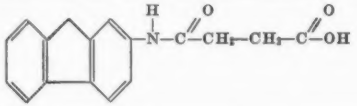
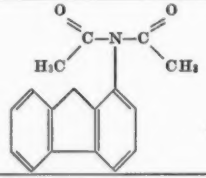
TABLE 1.—Structural formula and melting point of chemicals tested for carcinogenic potency

	Structural formula	Expt. No.	Compound	M.p. ° C.	Ref.
(a)*		192 192A	Fluorene	112–115 115.5–117	(15) (16)
		194	*N*-(7-chloro-2-fluorenyl)acetamide†	230	(16)
(b)		198	*N*-(7-iodo-2-fluorenyl)acetamide	224–225	(17)
		237	*N*-(3-iodo-2-fluorenyl)acetamide	206	(18)
		275	*N*-(2-fluorenyl)2,2,2-trifluoroacetamide‡	200–201	(19)
(c)		276	*N*-(2-fluorenyl)2-fluoroacetamide‡	168–169	(20)

See footnotes at end of table.

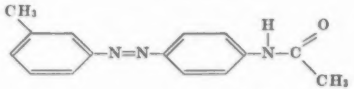
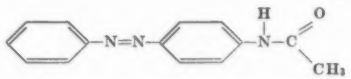
TABLE 1.—Structural formula and melting point of chemicals tested for carcinogenic potency—Continued

	Structural formula	Expt. No.	Compound	M.p. °C.	Ref.
		247	*N*-4-fluorenylacetamide	199–200	(*11*)
(*d*)		256	*N*-1-fluorenylacetamide	187–189	(*22*)
		266	*N*-3-fluorenylacetamide	188–189	(*23*)
		257	*N*-(9-oxo-2-fluorenyl)acetamide	224–225	(*24*)
(*e*)		274	*N*-(7-methoxy-2-fluorenyl)acetamide	204–206	(*25*)

		191	Pavatrine (commercial grade, G. D. Searle Co.)	—	—
		200	*N*-2-fluorenyl-*p*-toluenesulfonamide	162–163	(*86*)
(*f*)		195	2-Acetamido-4-(2′-fluoryl)thiazole†	304–305	(*87*)
		259	4,4′-Sulfonylbisacetanilide	291–293	(*88*)
(*g*)		236	*N*-2-fluorenylsuccinamic acid	224–225	(*89*)
(*h*)		258	*N*-1-fluorenyldiacetamide	135–136	(*23*)

See footnotes at end of table.

TABLE 1.—Structural formula and melting point of chemicals tested for carcinogenic potency—Continued

	Structural formula	Expt. No.	Compound	M.p. °C.	Ref.
(*l*)		261	*m*-Tolylazoacetanilide§, ‖	151–153	(30)
		262	*p*-Phenylazoacetanilide§, ¶	157–159	(30)

*Marginal letters represent the same structural types described on page 151.
†Assisted by S. Schulman.
‡Assisted by H. Bryant.
§Assisted by J. N. Little.
‖3′-Methyl-4-dimethylaminoazobenzene (3′-methyl DAB derivative).
¶4-Dimethylaminoazobenzene (DAB derivative).

TABLE 2.—Carcinogenic studies of rats fed various chemicals related to fluorene or azo dyes

Expt. No.	Compound	Number started / Effective No.*	Sex and average starting age (*months*)	Age at autopsy (*months*)		Months on compound		Percent of compound in diet	Average intake of compound per rat‡ (*mg.*)	
				Average	Range	Average	Range†		Daily	Total
			Control							
222§	—	18/18	F 1.8	13.9	7.8–18.2	—	—	—	—	—
222A‖	—	18/18	F 3.5	15.5	9.4–19.9	—	—	—	—	—
			(a) Absence of the nitrogen attached to the ring							
192–1‡	Fluorene	20/11	F 3.0	10.2	8.2–10.7	6.1	5.0–6.2	0.05	4.3	796
192–2‖, ¶	Fluorene	18/18	F 0.9	19.0	5.1–20.1	18.1	4.1–19.2	0.05	4.6	2553

(b) Halogenation on the ring										
194**, ††	*N*-(7-chloro-2-fluorenyl)acetamide	10/7	F 2.5	5.7	5.0–7.6	2.5	—	0.05	3.3	253
198**, ††	*N*-(7-iodo-2-fluorenyl)acetamide	20/16	F 2.2	9.7	4.5–22.2	4.7	2.6–6.2	0.05	3.7	535
237§, ‡‡	*N*-(3-iodo-2-fluorenyl)acetamide	18/16	F 3.8	13.6	5.1–16.7	9.9	2.1–12.5	0.025	2.2	676
(c) Halogenation on the side chain										
275§, ‡‡	*N*-(2-fluorenyl)2,2,2-trifluoroacetamide	18/18	F 3.3	13.5	11–15.8	10.2	7.8–12.2	0.027	2.4	751
276§, ‡‡	*N*-(2-fluorenyl)2-fluoroacetamide	2/2 7/7	M 1.7 F 1.9	8.9	5.9–11.8	7.0	4.1–9.4	0.031	2.2	459
(d) Positional isomers										
256§, ‡‡	*N*-1-fluorenylacetamide	18/17	F 2.2	17.2	6.7–22.5	10.3	4.5–11.0	0.025	2.8	874
266§, ‡‡	*N*-3-fluorenylacetamide	18/17	F 3.4	18.9	12.4–21.9	15.4	9.2–17.7	0.025	2.6	1217
247§, ‡‡	*N*-4-fluorenylacetamide	18/13	F 3.0	18.6	6.7–25.4	13.3	3.4–14.3	0.025	2.6	1048
(e) Oxidation or other changes at certain positions										
257§, ‡‡	*N*-(9-oxo-2-fluorenyl)acetamide	18/18	F 2.6	17.6	5.5–22.5	15.0	2.8–19.8	0.025	2.7	1215
274§, ‡‡	*N*-(7-methoxy-2-fluorenyl)acetamide	18/17	F 4.7	15.5	6.6–19.9	10.1	2.3–12.2	0.028	2.6	788
191**, ††	Pavatrine	20/11	F 4.0	10.3	8.3–11.8	5.7	4.3–6.2	0.05	4.2	736
(f) Addition of sulfur-containing substituents										
200**, ††	*N*-2-fluorenyl-*p*-toluenesulfonamide	10/4	F 3.0	8.7	4.9–10.6	5.1	1.9–6.2	0.05	4.0	625
195**, ††	2-Acetamido-4-(2′fluoryl)thiazole	10/4	F 4.9	12.4	11.5–13.5	6.2	—	0.05	4.6	865
259§, ‡‡	4,4′-sulfonylbisacetanilide	2/2 16/16	M 2.0 F 1.9	19.9	12.1–23.2	10.1	10.0–10.1	0.025	2.7	833

See footnotes at end of table.

TABLE 2.—Carcinogenic studies of rats fed various chemicals related to fluorene or azo dyes—Continued

Expt. No.	Compound	Number started / Effective No.*	Sex and average starting age (*months*)	Age at autopsy (*months*)		Months on compound		Percent of compound in diet	Average intake of compound per rat‡ (*mg.*)	
				Average	Range	Average	Range†		Daily	Total
	(g) Lengthening of the side chain									
236§, ‡‡	*N*-2-fluorenylsuccinamic acid	18/17	F 3.8	20.1	12.8–27.6	16.7	9.3–23.7	0.025	2.4	1241
	(h) Diacetylation									
258§, ‡‡	*N*-1-fluorenyldiacetamide	2/2 16/16	M 1.9 F 1.9	19.1	10.0–23.4	12.2	8.3–12.4	0.025	2.8	1039
	(i) Acetylation of phenylazoanilines									
261§, ‡‡	*m*-Tolylazoacetanilide	18/17	F 3.6	19.5	9.0–25.0	11.8	4.9–12.4	0.025	2.6	940
262§, ‡‡	*p*-Phenylazoacetanilide	18/17	F 3.6	20.4	12.9–25.4	12.0	9.2–12.4	0.025	2.6	939

*"Effective number" pertains to the number of animals whose tissues were satisfactory for microscopic examinations made.

†The maximum time represents the longest period any animal was fed the diet containing the particular compound.

‡The intake of each compound is based on the "effective number" of animals for each experiment. The daily intake value was calculated from the total intake of diet containing the compound, the percentage of compound in the diet, and the number of days the rats ingested the diet. The great variation in drug intake is caused by the variable length of time the animals ingested the drug-containing diet, rather than large variations in daily intake.

§Basal diet 222.

‖Basal diet 222A.

¶The chemical was dissolved in warm corn oil and the resulting solution, while still warm, was added to the other diet constituents and mixed as in **.

**The chemical was first dissolved in acetone and after thorough mixing with the cerelose constituent the solvent was removed at room temperature, in a hood. The dry-sugar mixture was then thoroughly incorporated into the diet mixture with a mechanical mixer.

††Basal diet 143.

‡‡The compound was dissolved in the warm propylene glycol portion of the diet, and this solution was immediately added to the other dietary constituents and mixed as in **.

younger than the animals started at the older age. The experimental animals were usually autopsied at the average age of 20.4 months. A few animals in several groups were older than 20 months by the time they were killed.

All rats in the experiments consumed a substantial amount of drug as indicated by the average daily intakes, table 2. This was influenced more by the level at which the compound was incorporated into the experimental diet rather than by its chemical structure; the average intake varied from 2.2 to 4.6 mg. per rat. The average total intake per rat not only reflects the level of incorporation into the diet but also the total length of the drug-feeding period, table 2.

(a) Removal of the Nitrogen Attached to the Ring

Experiments (192-1 and 192-2) were carried out with 2 highly purified samples of fluorene (table 1). The fluorene was incorporated into slightly different basal diets but at the same percentage level (0.05%).

Experiment 192-2 was nearly 3 times the length of the first experiment—19 *versus* 7 months, table 2(a)—and the total intake of fluorene was also over 3 times that in the first test. The number of tumors was essentially the same, but the location and types of tumors were different in the 2 experiments (table 3). The increased total intake of the chemical and a longer experimental period did not materially increase the number of neoplasms observed. A comparison of the tumors observed in a control group of Buffalo-strain female rats fed diet 222A—the same basal diet as in experiment 192-2 and for approximately the same period (table 3)—seems to support the view that fluorene has very slight, if any, carcinogenic activity when fed to the Buffalo-strain female rat, under the conditions of these tests.

(b) Halogenation on the Ring of Fluorenylacetamide

N-2-(7-chloro)fluorenylacetamide incorporated at a level of 0.05 percent of the diet proved toxic to female rats. Survival of 7 animals in this test ranged from 2.5 to 5 months [table 2(b), expt. 194]. One rat developed multiple hepatomas after 3 months. Cirrhosis of the liver occurred in 4 rats, 1 developed a hemangioma, and 1 showed early parenchymal degeneration with focal periportal bile-duct hyperplasia (table 3).

Owing to the small quantity of material available for testing, it was not possible to repeat the test with a lower level of feeding over a longer period, or in male rats, in an attempt to study the carcinogenic activity. It must be concluded that this 7-chloro derivative is carcinogenic, but additional tests are needed to evaluate more fully the carcinogenic potency.

The (7-iodo)-2-fluorenylacetamide compound was fed at a level of 0.05 percent for 6 months, and the average age at autopsy was 9.7 months (table 2). One rat 18 months of age developed a chromophobe-type adenoma of the pituitary. Since an occasional pituitary adenoma occurs

TABLE 3.—Tumors and other lesions in rats fed diets containing the various chemicals listed (*see also* table 2)

Compound tested	Number of rats	Neoplastic reactions			Non-neoplastic reactions		
		Area or organ	Type of tumor	Number of animals	Area or organ	Type of reaction	Number of animals
Control diet 222A	18	Uterus	Adenocarcinoma	1	Large intestine	Epithelial ulcer, chronic	1
		Uterus	Fibro-epithelial-polyp	2			
		Adrenal	Cortical adenoma	5			
		Pituitary	Adenoma	6			
		Inguinal region	Fibroma	1			
Control diet 222	18	R-E system	Granulocytic leukemia	1			
		Kidney	Adenoma	1			
		Pituitary	Adenoma, chromophobe type	2			
(a) Absence of nitrogen attached to the ring							
Fluorene-1	11	Kidney	Squamous-cell carcinoma, noninvasive, pelvis	1	Forestomach	Acanthosis, hyperkeratosis	5
		Ureter	Squamous-cell carcinoma, noninvasive	1	Kidney	Squamous metaplasia, pelvis	7
					Uterus	Squamous metaplasia	1
					Small intestine	Epithelial ulcer, acute	1
					Liver	Cirrhosis	3
Fluorene-2	18	Uterus	Fibrosarcoma	1	Pituitary	Hyperplasia, predominantly chromophobe cells	2
		Uterus	Carcinosarcoma	1			
		R-E system	Granulocytic leukemia	1			
		Pituitary	Adenoma, chromophobe type	4			
(b) Halogenation on the ring							
N-(7-chloro-2-fluorenyl)acetamide	7	Liver	Hepatoma	1	Liver	Portal cirrhosis	4
		Liver	Hemangioma	1	Liver	Nonspecific parenchymal degeneration with focal bile-duct ectasia	1
					Forestomach	Hyperkeratosis	2
N-(7-iodo-2-fluorenyl)acetamide	16	Pituitary	Adenoma, chromophobe type	1	Stomach	Acanthosis and hyperkeratosis	10
					Pituitary	Hypertrophy	2
					Liver	Fatty degeneration	10
					Liver	Cyst	1
					Liver	Portal cirrhosis	3
					Liver	Necrosis	2
					Thyroid	Hyperplasia	5
					Brain	Abscess	3

N-(3-iodo-2-fluorenyl)acetamide	16	External auditory canal	Squamous-cell carcinoma* 6 bilateral	8	Forestomach	Hyperkeratosis and acanthosis	1
		Uterus	Adenocarcinoma	1	Adrenal	Cortical nodule	1
		Mammary	Adenocarcinoma	1	Harderian gland	Hypertrophy	1
		Lung	Adenoma	1			
		Lung	Adenocarcinoma	1	Brain	Abscess	2
		R-E system	Reticulum-cell sarcoma	1			
		R-E system	Leukemia, indeterminant type	2			
			(c) Halogenation on the side chain				
N-(2-fluorenyl)2,2,2-trifluoroacetamide	18	Mammary†	Adenocarcinoma	14	Small intestine	Glandular hyperplasia	1
		Mammary	Fibrosarcoma	1			
		Mammary	Fibroma	1			
		Mammary	Carcinosarcoma	1	Forestomach	Acanthosis, hyperkeratosis, and papillomatosis	12
		Mammary	Squamous-cell carcinoma	1			
		Liver	Hepatoma	13			
		External auditory canal	Squamous-cell carcinoma, 1 bilateral	7	R-E system	Leukemoid hyperplasia	
		Adrenal	Cortical adenoma	1			
		Uterus	Sarcoma	1			
		Small intestine	Adenocarcinoma	2			
		Forestomach	Squamous-cell carcinoma	2			
		R-E system	Granulocytic leukemia	2			
N-(2-fluorenyl)2-fluoroacetamide	9	Liver	Hepatoma	1	Forestomach	Acanthosis, hyperkeratosis, and papillomatosis	4
		Muscle	Hemangioma	1	Heart	Endocarditis, periarteritis	1
			(d) Positional isomers of *N*-2-fluorenylacetamide				
N-1-fluorenylacetamide	17	Ovary	Granulosa-cell carcinoma	1	Large intestine	Ulcer, chronic	2
		Uterus	Carcinosarcoma	1	Large intestine	Atypical glandular hyperplasia	1
		Adrenal	Carcinoma	1	Forestomach	Hyperkeratosis	3
		Pituitary	Adenoma	3	Thyroid	Hyperplasia	1
		R-E system	Leukemia, indeterminant type	1	Mammary	Atypical glandular hyperplasia	1
					Glandular stomach	Ulcer	1
N-3-fluorenylacetamide	17	Uterus	Adenocarcinoma	1	Forestomach	Acanthosis, papillomatosis, hyperkeratosis	5
		Kidney	Adenoma	1			
		Liver	Hepatoma	1	Forestomach	Ulcer, acute	1
		R-E system	Granulocytic leukemia	1	Adrenal	Cortical nodule	1
		Thyroid	Adenoma	2	Lung	Squamous metaplasia	1
		Pituitary	Hemangioma	2			
N-4-fluorenylacetamide	13	Uterus	Sarcoma type—indeterminant (myxo- or liposarcoma)	1			
		R-E system	Leukemia, indeterminant type	1			
		Mammary	Adenofibroma	1			

See footnotes at end of table.

TABLE 3.—Tumors and other lesions in rats fed diets containing the various chemicals listed (*see also* table 2)—Continued

Compound tested	Number of rats	Neoplastic reactions			Non-neoplastic reactions		
		Area or organ	Type of tumor	Number of animals	Area or organ	Type of reaction	Number of animals
		(e) Oxidation or other changes of certain positions					
N-(9-oxo-2-fluorenyl)acetamide	18	Mammary‡	Adenocarcinoma	11			
		Mammary	Carcinosarcoma	1			
		Liver	Hepatoma	1			
		Uterus	Adenocarcinoma	2			
			Fibrosarcoma	1			
			Papillary adenocarcinoma	1			
		External auditory canal	Squamous-cell carcinoma	3	Forestomach	Ulcer, mucosa	1
		Lymph nodes	Lymphosarcoma	2	Lymph nodes	Reticulum-cell hyperplasia	1
		R-E system	Leukemia, indeterminant type	1			
		Kidney	Adenoma	4			
		Pancreas	Ductal carcinoma	1			
		Pituitary	Adenoma	1			
N-(7-methoxy-2-fluorenyl)acetamide	17	Mammary§	Adenocarcinoma	7	Large intestine	Ulcer, chronic	1
		External auditory canal	Squamous-cell carcinoma	3	Forestomach	Acanthosis and hyperkeratosis	5
		Small intestine	Adenocarcinoma	1	Spleen	Leukemoid hyperplasia	2
		Thyroid	Adenocarcinoma	1			
		R-E system	Granulocytic leukemia	1			
		Lung	Adenocarcinoma	1			
Pavatrine	11	—	—	—	Liver	Portal cirrhosis	5
					Kidney	Squamous metaplasia, pelvis	3
					Forestomach	Hyperkeratosis, papillomatosis	2
		(f) Addition of sulfur-containing groups					
N-2-fluorenyl-*p*-toluenesulfonamide	4	Uterus	Adenocarcinoma	1	Forestomach	Acanthosis and hyperkeratosis	2
					Forestomach	Focal area of cellular atypism	2
					Liver	Nodular regeneration	1
					Pituitary	Hyperplasia, slight	1
2-Acetamido-4-(2′-fluoryl) thiazole	4	Adrenal	Adenoma	1	Liver	Early portal cirrhosis	2
4,4′-Sulfonylbisacetanilide	18	Uterus	Adenocarcinoma	1	Forestomach	Acanthosis and hyperkeratosis	2
		Mammary	Adenocarcinoma	1	Small intestine	Lymphoid hyperplasia	1
		Pancreas	Fibrosarcoma	1	Liver	Cirrhosis	1
		R-E system	Leukemia, indeterminant type	2			
		Pituitary	Adenoma	3			

			(g) Lengthening of side chain				
N-2-fluorenylsuccinamic acid	17	Liver	Hepatoma	6	Forestomach	Acanthosis and hyperkeratosis	2
		Liver	Lymphangioma	1	Harderian gland	Hypertrophy	1
		Mediastinum	Fibrosarcoma	1			
		Uterus	Adenocarcinoma	1	Liver	Nodular regeneration	2
		R-E system	Reticulum-cell sarcoma	1	Adrenal	Cortical nodule	1
		Mammary	Gross tumor‖	1	Pituitary	Hyperplasia	1
		R-E system	Lukemia, indeterminant type	2			
			(h) Diacetylation				
N-1-fluorenyldiacetamide	18	Mammary	Adenocarcinoma	3	Forestomach	Acanthosis, hyperkeratosis	2
		Pituitary	Adenoma	7	Adrenal	Cortical nodule	1
		Uterus	Fibrosarcoma	1	Spleen	Leukemoid hyperplasia	1
		Liver	Hemangioma	1			
		External auditory canal	Squamous-cell carcinoma	1			
		Lymph node	Lymphosarcoma	1			
			(i) Acetylation of phenylazoanilides				
m-Tolylazoacetanilide	17	Uterus	Adenocarcinoma	1	Adrenal	Cortical nodule	2
		Uterus	Fibrosarcoma	1	Small intestime	Ulcer, chronic	3
		Mammary	Gross tumor‖	1	Liver	Early cirrhosis	3
		Thyroid	Adenoma	1			
		R-E system	Granulocytic leukemia	1	Lymph nodes	Reticulum-cell hyperplasia	1
		Adrenal	Cortical adenoma	1			
		Pituitary	Adenoma	2			
p-Phenylazoacetanilide	17	Mammary	Adenocarcinoma	1	Liver	Cirrhosis, early	1
		Middle ear	Fibrosarcoma	1	Forestomach	Hyperkeratosis	3
		External auditory canal	Gross tumor¶	1	Large intestine	Ulceration	1
		Uterus	Fibrosarcoma	1	Adrenal	Cortical nodule	1
		R-E system	Reticulum-cell sarcoma	1	R-E system	Leukemoid reaction	1
			Granulocytic leukemia	2			
		Pituitary	Adenoma	3			
		Intra-abdominal	Fibrosarcoma, primary site undetermined, with metastases	1			

*Eight rats with 14 ear-duct tumors.

†Fourteen rats with 32 mammary tumors.

‡Eleven rats with 24 mammary tumors.

§Seven rats with 12 mammary tumors—1 additional subcutaneous tumor included as a mammary tumor was not examined microscopically.

‖One subcutaneous tumor (gross).

¶One ear-duct tumor noted (gross).

spontaneously in this strain of rat at this age, it is highly unlikely that the chemical caused this neoplasm. A large number of non-neoplastic lesions were noted in the stomach, liver, thyroid, and brain (table 3). Only 4 of the 16 rats were autopsied after they reached 1 year or more. Most of the animals in the experiment were killed after 3 to 5 months; the lesions observed in these animals did not appear different in extent or location from those in the older animals that had been taken off the drug for several months before they were killed. The semisynthetic diet used may have been a contributing factor to the occurrence of the numerous other lesions, since some liver cirrhosis and squamous metaplasia of the kidney were found in animals ingesting the same basal diet, but which contained the noncarcinogenic drug Pavatrine—*see* later.

The feeding of a diet, 222, containing 0.025 percent of 3-iodo-2-fluorenylacetamide resulted in the development of a high incidence of squamous-cell carcinomas of the ear duct—14 in 8 rats. Also a few other tumors developed. The rats averaged 3.8 months of age (table 2) at the start of the experiment. The very low incidence of mammary-gland tumors may be related to this older starting age, since it is known that fewer mammary-gland tumors develop when old rats are started on diets containing 2-FAA (*13, 31, 32*). The rats developing ear tumors were on the experiment from 10 to 12.5 months, averaging 11.8 months. They received the 3-iodo-2-FAA continuously until killed.

(c) Halogenation on the Side Chain

Replacing all the hydrogens on the ω-carbon atom of the acetyl group with the halogen fluorine resulted in a very potent carcinogen. When that compound, *N*-2-(fluorenyl)2,2,2-trifluoroacetamide, was fed to 18 rats at a level of 0.027 percent of the diet for a maximum of 12.2 months (table 2), a large number of adenocarcinomas of the mammary gland, hepatomas, squamous-cell carcinomas of the ear duct, and also tumors of several other sites were observed (table 3). These rats averaged 3.3 months of age when started on the experiment. They ingested the carcinogen for an average of 10.2 months, from 7.8 to 12.2 months (table 2). Because of the high incidence of tumors, many rats had to be killed before the end of the 12th month of their being fed the carcinogen. Other lesions appearing were papillomatosis, acanthosis, and hyperkeratosis of the forestomach, and glandular hyperplasia of the small intestine (table 3).

Replacing only one of the hydrogens on the ω-carbon atom of the acetyl group apparently decreased carcinogenicity when the compound was fed at a level of 0.031 percent (table 2, expt. 276). Only 2 tumors and a few other lesions were noted among several rats (table 3); one rat had subacute endocarditis and periarteritis. The animals survived for a shorter period than in the experiment in which 3 hydrogens had been replaced by fluorine, and the monofluoro compound appeared to be more toxic.

(d) Positional Isomers of *N*-2-Fluorenylacetamide

N-1-fluorenylacetamide (1-FAA) was fed for 11 months at 0.025 percent of the diet. The rats surviving 11 months were kept under observation for a longer period after they were put on the control diet 222. Seven tumors including 1 leukemia and 3 pituitary adenomas were noted. The latter developed in rats at 20, 20, and 19 months of age, respectively (table 3). The average total intake of 1-FAA per rat was 0.87 gm. (table 2). Several other lesions were observed, table 3—forestomach hyperkeratosis in 3; 1-FAA at the level fed in this test was only slightly carcinogenic.

N-3-fluorenylacetamide (3-FAA) was fed at 0.025 percent of the diet for 17.7 months. At that time, 7 of the 18 rats started on the diet were still living (table 2, expt. 266); 17 of the 18 animals ingested 3-FAA for 10 months. The duration of the experiment was 9.2 to 17.7 months, with an average of 15.4 months. Four of the 17 rats autopsied had tumors. One rat, 14.5 months of age, on 3-FAA for 9 months, developed a hepatoma, an adenocarcinoma of the uterus, and a kidney adenoma; 1 animal, 14.5 months old, had leukemia, 2 had pituitary hemangiomas, and 2, 20.5 and 21.5 months of age, had thyroid adenomas. Thyroid tumors are not known to occur in this strain of rat spontaneously (table 3). Other lesions were observed in the forestomach, adrenal, and lung (table 3). Under the conditions of this experiment, 3–FAA was not a potent carcinogen. Although the average total intake of 3-FAA per rat was 1.2 gm., a value higher than that of some other chemicals because of the long feeding period (table 2), it should be tested at a higher dietary concentration to determine whether a still higher intake would increase carcinogenic activity.

After diet 222 containing 0.025 percent of *N*-4-fluorenylacetamide (4-FAA) was fed for an average of 13.3 months, the average total intake of the drug per rat was 1.05 gm. In 13 of the 18 rats started on the diet, tumors were found in 3, as follows: 1 myxosarcoma or liposarcoma of the uterus in a rat 19 months of age, 1 leukemia in a 19-month-old rat, and 1 adenofibroma of the mammary gland in a 22-month-old rat (table 3). The histological characteristics of the latter tumor had not changed from that of a biopsy made 5 months earlier; it was probably of spontaneous origin. Although 4-FAA appears to have slight carcinogenic activity, another test of a diet containing a higher percentage of the chemical should be tried.

(e) Oxidation or Other Changes at Certain Positions

Eighteen female rats, 2.5 months old, were fed *N*-(9-oxo-2-fluorenyl)-acetamide in diet 222 at a level of 0.025 percent, for an average of 15 months. The average total intake per rat was 1.2 gm. (table 2, expt. 257). The duration of the experiment was 3 to 20 months, with an average of 15 months. Twenty-two adenocarcinomas and 1 carcinosarcoma of the mammary gland were noted in 11 rats, 7 to 20 months of age (table 3). One rat 20 months of age had a hepatoma; 3 squamous-

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cell carcinomas of the ear duct were observed in 3 rats; and also 2 adenocarcinomas, 1 fibrosarcoma, and 1 papillary carcinoma of the uterus were noted. Four adenomas of the kidney tubules were observed in 4 rats. Two lymphosarcomas were noted in rats 18 and 19 months of age; 1 rat 18 months of age developed leukemia. One carcinoma of the pancreas was observed in a rat 17 months of age and 1 pituitary adenoma in a 20-month-old rat. A total of 42 tumors was observed in 16 rats. Only 2 rats, killed after 3 months on the diet, were without tumors. In this test, *N*-(9-oxo-2-fluorenyl)acetamide, although very carcinogenic at several sites, was only slightly carcinogenic to the liver, since only 1 rat developed a hepatoma. The complete study of the microscopic material from this series of animals shows many more tumors than those originally mentioned in the preliminary report (*1*), though no additional liver tumors were discovered.

N-(7-methoxy-2-fluorenyl)acetamide, when fed in diet 222 at a level of 0.028 percent for 1 year to female rats, proved to be carcinogenic (table 2, expt. 274). These animals were 4.7 months of age when started; nevertheless, 11 mammary-gland adenocarcinomas developed in 6 rats, and 1 rat developed a subcutaneous tumor (probably mammary), which was not examined microscopically (table 3). Tumors of several other sites were noted, including the ear duct, the small intestine, the thyroid, the lung, and 1 granulocytic leukemia, table 3. Tumors developed in 10 rats. The average total intake of the chemical per rat for the 1-year period was 0.79 gm. Other lesions developed in the forestomach, the large intestine, and the spleen. Blocking the 7-position with a methoxy group decreased somewhat the carcinogenic potency of 2-FAA, but did not prevent the induction of a large number and variety of tumors. No liver tumors developed in the female rats under the conditions of this test, though tumors at 6 other sites were noted.

Another change at the 9-position was tested when the antispasmodic drug, Pavatrine, was included in the diet at a level of 0.05 percent for a period of 5.7 months (table 2). This drug contains no amide nitrogen attached to the ring. The nitrogen is a part of a long side-chain attached at the 9-position. No gross or microscopic tumors were found. Microscopic study was made on 11 of 20 rats started on the diet. Liver cirrhosis, squamous metaplasia of the kidney pelvis, and hyperkeratosis and papillomatosis of the forestomach were noted, table 3.

(f) Sulfur-Containing Substituents

Tissues were examined microscopically in 4 of 10 rats that ingested a diet containing 0.05 percent *N*-2-fluorenyl-*p*-toluenesulfonamide for an average of 5.1 months (table 2). Only 1 rat 8 months of age developed a microscopic tumor, an early adenocarcinoma of the uterus (table 3), which is a not too uncommon tumor in this strain. No gross tumors appeared. Other lesions noted included hyperkeratosis and acanthosis with cellular atypism of the forestomach and nodular regeneration of the

liver. The rat with the uterine tumor also had the lesions in the liver, ovary, and stomach. The average total drug intake per rat was nearly 0.63 gm. (table 2). Nine of the 10 rats, averaging 3.0 months of age when started on the diet, survived beyond 27 weeks, but all had been killed by the 34th week. It must be concluded that *N*-2-fluorenyl-*p*-toluenesulfonamide was essentially inactive as a carcinogen in this study.

In 1 of 4 rats whose tissues were examined microscopically after ingesting a diet containing 0.05 percent of 2-acetamido-4-(2′-fluoryl)thiazole, an adrenal adenoma was observed. This rat was 14 months of age when killed. Two other rats developed early cirrhosis of the liver (table 3). The animals averaged 4.9 months of age at the start, and 9 of the 10 rats survived the 6-month period during which the diet containing the chemical was fed. The average total intake per rat for this period amounted to 0.86 gm. One rat survived until 27 months of age, but died without any gross tumors (table 2).

Another sulfur-containing derivative, 4,4′-sulfonylbisacetanilide, was fed at a level of 0.025 percent in diet 222 to 17 females and 1 male (table 2). The animals were 2 months of age when started and they ingested an average total of 0.83 gm. of the chemical over a period of 10 months. Microscopically, we found 1 fibrosarcoma of the pancreas, 1 adenocarcinoma of the uterus, 1 adenocarcinoma of the mammary gland, pituitary adenomas in 3, and also leukemia in 2 rats (table 3). One other lesion, a lymphoid hyperplasia of the intestine, was observed in a rat 12.5 months of age. No tumors were found in 13 of the 18 rats, while a tumor was seen in each of the other 5 animals. The sulfur-containing 4,4′-sulfonylbisacetanilide was somewhat carcinogenic in this test.

(g) Effect of Long Side-Chains

One long side-chain compound tested was *N*-2-fluorenylsuccinamic acid. This compound has been reported to be carcinogenic, but no details concerning the types or number of tumors induced have been described (*29*). Of 18 rats started, 4 killed under 1 year were without tumors. Eleven animals developed neoplasms after ingesting a diet containing 0.025 percent of the chemical for 9.3 to 23.7 months. These neoplasms were described as follows (table 3): 6 rats with hepatoma, 1 with lymphangioma of the liver, 1 with fibrosarcoma of the mediastium, 1 with adenocarcinoma of the uterus, 1 with mammary tumor (from gross observation) and also a lymphangioma, 1 with reticulum-cell sarcoma, and 2 rats with leukemia. The average total intake per rat was 1.24 gm. Two rats killed after 17 to 18 months showed nodular regeneration in the liver. Other nontumorous lesions were noted in the adrenal, the pituitary, the forestomach, and the harderian gland. It must be concluded from this test that *N*-2-fluorenylsuccinamic acid is an active carcinogen. The almost complete absence of tumors of the mammary glands may have been partially due to the older starting age (*13*, *31*, *32*) of the rats, which ranged from 3.5 to 5.0 months, averaging 3.8.

(h) Diacetylation

The striking effect of replacing the second hydrogen on the nitrogen with an acetyl group as in *N*-2-fluorenyldiacetamide (*3*) led to the study of the carcinogenic properties of *N*-1-fluorenyldiacetamide. The results are presented in tables 2 and 3, experiment 258. Sixteen females and 2 males starting at approximately 2 months of age were fed diet 222 containing 0.025 percent *N*-1-fluorenyldiacetamide for 12 months. The average total intake of the chemical per rat was 1.04 gm. No tumors were found in the 2 males and in 7 females. In the 9 female rats with tumors, we found adenocarcinoma of the mammary gland, pituitary adenomas, a squamous-cell carcinoma of the ear duct, fibrosarcoma of the uterus, hemangioma of the liver, and 1 lymphosarcoma of a lymph node. Other lesions noted were forestomach hyperkeratosis or acanthosis in 2 rats and an adrenocortical nodule on 1 rat. The diacetylation of *N*-1-fluorenamine increased slightly the carcinogenic potency over monoacetylation. Only 1 liver tumor, however, and very few mammary tumors developed. Both mono- and diacetyl-derivatives of 1-fluorenamine are much less carcinogenic than the corresponding acetyl derivatives of 2-fluorenamine (*2*, *3*).

(i) Acetylation of Certain Azo Dyes

Two acetylated azo dyes, *m*-tolylazoacetanilide and *p*-phenylazoacetanilide, were fed, for 12 months, in diet 222, each at a level of 0.025 percent. The rats averaged 3.6 months of age at the start of the feeding period and were kept under observation for approximately 6 months after the cessation of carcinogen feeding (tables 2 and 3, expts. 261 and 262). No liver tumors developed, which is corroborated by many investigators (*4*) who find that methyl groups attached to the nitrogen are essential for liver carcinogenesis with these 2 azo compounds. The acetylated derivatives in these tests, however, were slightly carcinogenic. A few tumors developed at a number of sites distant from the portal of entry. It is well known that the dimethyl derivatives of these 2 compounds are liver-specific carcinogens and it is interesting to find that after acetylation of these 2 compounds, tumors at other sites can be produced. Thus, after acetylation, these 2 azo dyes become more like weak carcinogens of the 2-FAA type.

Eight tumors at 7 sites were identified in rats ingesting *m*-tolylazoacetanilide (table 3) and 10 tumors at 8 sites in animals ingesting a diet containing *p*-phenylazoacetanilide.

Summary of Carcinogenic Activity

Those compounds inducing a single liver tumor did not result in any mammary tumors and those compounds in which a single mammary tumor developed did not result in any liver tumors (table 4).

N-(2-fluorenyl)-2,2,2-trifluoroacetamide was active in inducing tumors of the liver, mammary gland, and ear duct (table 4). No other com-

TABLE 4.—Summary of carcinogenic activity of those compounds in which there appeared one or more tumors of the liver, mammary gland, or ear duct

Compound	Number of animals	Liver			Mammary gland			Ear duct		
		Slightly active	Moderately active	Most active	Slightly active	Moderately active	Most active	Slightly active	Moderately active	Most active
N-7-chloro-2-FAA	7	2								
N-(9-oxo-2-fluorenyl) acetamide	18	1					23		4	
3-FAA	17	1								
N-(2-fluorenyl)-2-fluoro AA	9	1								
N-2-fluorenylsuccinamic acid	17		6		1					
N-(2-fluorenyl)-2,2,2-trifluoro AA	18			13			32			8
N-(3-iodo)-2-FAA	16				1					14
1-FAA	17				1					
1-FdiAA	18					3		1		
4,4'-Sulfonylbisacetanilide	18				1					
m-Tolylazoacetanilide	17				1					
p-Phenylazoacetanilide	17				1					
N-(7-OCH_3)-2-FAA	17						12		3	

pound tested was highly active for all these sites. 9-Oxo-2-FAA and 7-OCH_3-2-FAA were active in inducing mammary tumors and moderately active in inducing ear-duct tumors, but 3-iodo-2-FAA was highly active only in induction of ear-duct tumors.

The lengthening of the side-chain as in *N*-2-fluorenylsuccinamic acid almost completely eliminated carcinogenic activity of the mammary gland, but a substantial number of liver tumors developed in female Buffalo-strain rats. These animals averaged 3.8 months of age when started on the diet (table 2), the rats receiving *N*-(2-fluorenyl)2,2,2-trifluoroacetamide averaged 3.3 months, the rats in the group receiving 9-oxo-2-FAA and 7-OCH_3-2-FAA averaged 2.6 and 4.7 months of age when started, so it would appear that age *per se* does not preclude mammary-gland carcinogenesis.

Five types of tumors were noted in 36 control rats (table 5): 8 pituitary and 5 adrenal adenomas, 1 kidney tumor, 1 tumor of the uterus, and 1 leukemia. The Buffalo-strain female seems especially susceptible to development of adenomas of the pituitary and the adrenal glands. These tumors usually occur more frequently in older animals. The youngest control rat to develop a pituitary tumor was 14.0 months old and the youngest to develop an adrenal tumor was 13.3 months. Most rats in the experimental groups developing either pituitary or adrenal tumors were 15 or more months of age. The percentage of rats developing pituitary tumors, in the group ingesting 1-FdiAA, was about twice that for control animals (table 5). Four of these rats were over 20 months of age, but the oldest control animal was only 20 months. Because of this age difference it is difficult to attach a high degree of significance to the high incidence of pituitary tumors in rats receiving 1-FdiAA, though it merits further examination. The rats developing pituitary and adrenal tumors as presented in table 5 show no striking difference in age between control and experimental animals.

TABLE 5.—"Other tumors"* summary divided according to site and frequency of appearance

	Number of rats	Number and type of tumor†													
		Small intestine	Kidney	Uterus	Ureter	Ovary	Pancreas	Pituitary	Adrenal	Thyroid	Lymph node	Leukemia	Stomach	Lung	Sarcoma
Control	36		1	1				8 [a]	5 [b]			1			
2-AA-4-(2-fluoryl)thiazole	4								1 [c]						
N-(7-Iodo)-2-FAA	16			1											
Fluorene	29		1	2				4 [d]				1	1		
N-2-Fluorenylsuccinamic acid	17			1	1							2			2
N-(3-Iodo)-2-FAA	16			1								2		2	1
1-FAA	17			1		1		3 [e]	1 [f]						
1-FdiAA	18			1				7 [g]			1				
3-FAA	17		1	1				2 [h]		2		1			
N-(7-OCH_3)-2-FAA	17	1								1		1		1	
N-(2-F)2,2,2-trifluoro AA	18	2		1					1 [i]			2	2 (Fore)		
m-Tolylazoacetanilide	17			2				2 [j]	1 [k]	1		1			
p-Phenylazoacetanilide	17			1				3 [l]				2			2
4,4'-Sulfonylbisacetanilide	18			1				3 [m]				2			1
N-(9-oxo-2-fluorenyl)acetamide	18		4	4			1	1 [n]			2	1			

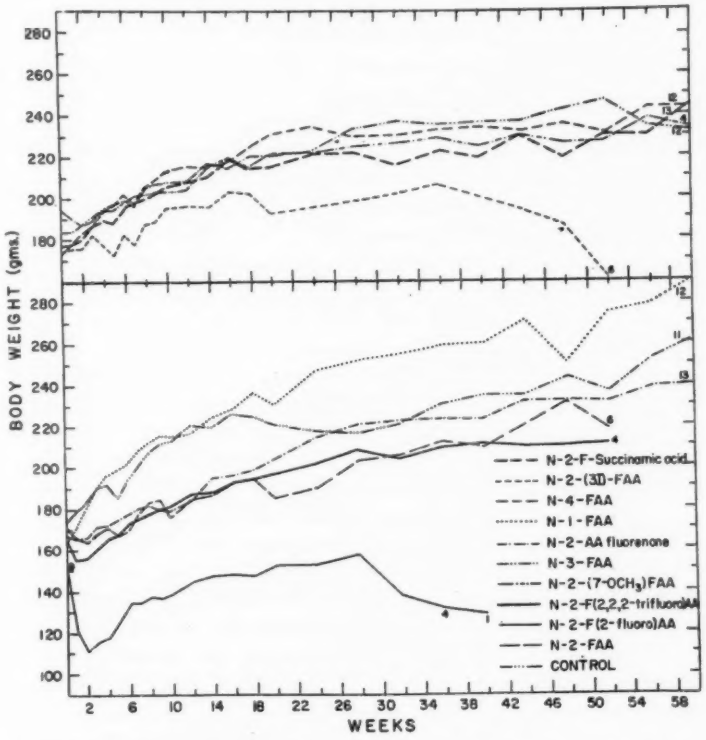
*"Other tumors" refers to tumors occurring at sites other than the liver, mammary gland, or ear duct.

†Age at autopsy:

a. 14.0, 15.7, 16.0, 18.0, 19.6, and 19.8 months: 2 adenomas in each of 2 rats.
b. 13.3, 15.7, 15.7, 19.6, and 19.8 months.
c. 13.5 months.
d. 17.5, 20.0, 20.0, and 20.1 months.
e. 19.9, 20.4, and 21.8 months.
f. 19.5 months.
g. 15.3, 17.4, 18.1, 22.2, 22.9, 23.1, and 23.4 months.
h. 17.6 and 21.3 months.
i. 13.2 months.
j. 21.9 and 22.3 months.
k. 15.3 months.
l. 20.7, 21.9, and 24.5 months.
m. 21.2, 23.1, and 23.2 months.
n. 22.3 months.

9-Oxo-2-FAA was the most active carcinogen for "other type" tumors. Six different types were noted in this group of animals. One to 5 "other type" tumors were noted in groups receiving other compounds (table 5).

The average body weights of 11 groups have been plotted in text-figure 1. The inclusion of all test groups in this summary would only have increased the complexity without aiding in the interpretation of the data as given. Each group was initially comprised of 18 female rats except the group receiving *N*-(2-fluorenyl)2-fluoroacetamide, which had 9 females. Four groups lost weight during the first 2 experimental weeks, namely, 7-OCH_3-2-FAA, 2-FAA, *N*-(2-fluorenyl)2,2,2-fluoro AA, and *N*-2-(fluorenyl)2-fluoro AA. After this initial loss, the rats began to gain in weight and those in 3 of the 4 groups had regained their initial weight by the end of the 4th week. Their average weights increased progressively thereafter for many weeks. The animals in the fourth group, ingesting *N*-(2-fluorenyl)2-fluoro AA, did not fully regain



TEXT-FIGURE 1.—Average body weight of 11 groups of rats ingesting the chemicals listed. Weights are plotted at bi-weekly intervals. Numbers on curves near *right* margin refer to number of animals alive at that time interval and used to compute the average weight. Chart is divided into two sections to avoid an excessive number of overlapping curves.

their average initial weight until after the 20th week. The rats in this latter group, moreover, developed few tumors.

The animals ingesting 7-OCH_3-2-FAA, and *N*-(2-fluorenyl)2,2,2-fluoro AA developed many tumors at various sites. There is no clear-cut evidence in these experiments that loss in average weight is a criterion of carcinogenicity when this loss occurs during the first few weeks of the experiment.

There was no initial average weight loss in animals ingesting *N*-(9-oxo-2-fluorenyl)acetamide, a chemical that induced many mammary tumors, *N*-2-fluorenylsuccinamic acid, a compound producing many hepatomas, or 3-iodo-2-FAA, a chemical inducing many ear-duct tumors; the greatest initial loss in average weight and the poorest recovery from this weight loss was caused by the non- or slightly carcinogenic chemical *N*-(2-fluorenyl)2-fluoro AA.

DISCUSSION

Carcinogenic polycylic hydrocarbons were reported, by Haddow *et al.* (*33*), to produce an immediate, constant, and long-continued reduction in the rate of growth of young rats, but a number of chemically similar noncarcinogenic hydrocarbons did not cause this inhibitory effect on body weight. Other treatments, including X radiation, lead, and colchicine, produced no effect or a temporary depression in weight followed by recovery to the normal growth rate. In contrast to the observations of Haddow *et al.* (*33*), a slight reduction in body weight was noted during the first 2 weeks for rats ingesting the highly carcinogenic chemicals 2-FAA, 7-OCH_3-2-FAA, and *N*-(2-fluorenyl)2,2,2-fluoro AA (text-fig. 1). However, no initial loss in weight occurred in rats ingesting *N*-2-fluorenylsuccinamic acid, a compound especially carcinogenic to the liver, or in rats ingesting *N*-(9-oxo-2-fluorenyl)acetamide, a compound highly carcinogenic to the mammary gland, or in those ingesting 3-iodo-2-FAA, a compound principally carcinogenic to the ear duct. Most of the groups whose weights have been plotted in text-figure 1 continued to increase in weight after this initial loss, or when there was no initial loss from the beginning of the test. The average body weight of some of the groups were above and some were below but they all paralleled the curves of control animals ingesting only a basal diet. However, of the 2 groups having the most prolonged reduction in weight those ingesting 3-iodo-2-FAA and *N*-(2-fluorenyl)2-fluoro AA, the former was carcinogenic and the latter only slightly carcinogenic. Body-weight changes, therefore, were not reliable criteria of carcinogenic activity.

Experiments on the metabolism of 2-FAA (*34*) indicate that the principal urinary metabolites are formed by hydroxylation at several ring positions and subsequent conjugation mostly with glucuronic acid, and a relatively small portion with sulfuric acid. The carcinogenic succinamic acid derivative could also be metabolized similarly to *N*-2-FAA if the desuc-

cinylation took place before hydroxylation and conjugation. It is less clear why the trifluoro derivative, *N*-(2-fluorenyl)-2,2,2-trifluoro AA, would be more carcinogenic than the *N*-(2-fluorenyl)-2-fluoro AA. The trifluoroacetyl derivative is much more soluble than the acetyl, or monofluoroacetylaminofluorene. One could assume that the trifluoro group is more readily hydrolyzed enzymatically to form the fluorenamine than the monofluoro group. If that occurred then its carcinogenic activity would be expected to be similar to that of fluorenamine, but if the monofluoroacetyl group was not readily separated from the nitrogen of fluorenamine, it might not be readily absorbed or if absorbed it might be more slowly metabolized or the toxicity of liberated monofluoracetate might well militate against tumor induction. Poorer absorption has been found for *N*-2-fluorenyl-*p*-toluenesulfonamide with 90 percent of the radioactivity, of an orally administered dose labeled with S^{35} in the side-chain, eliminated unchanged through the gastrointestinal tract (*35*). Less than 1 percent of the radioactivity of the dose, moreover, appeared in the urine in the form of fluorenamine. The slow rate of metabolism of the tosylamino bond was advanced (*35*) as one explanation for the noncarcinogenic activity of this compound, although the level of radioactivity in the liver 24 hours after administration was as high as that found 36 hours after oral administration of a comparable dose of 2-FAA (*36*). The liver activity, however, was not quite as high as that found 24 hours after oral administration of *N*-(7-hydroxy)-2-fluorenylacetamide (*37*).

Oxidation of *N*-2-FAA at the 9-position to form *N*-(9-oxo-2-fluorenyl)AA resulted in a compound which induced a wide variety of tumors at many sites, a carcinogenic activity greater for this compound than that reported by Miller *et al.* (*7*). These investigators showed that 6 of 9 female rats, when fed *N*-(9-oxo-2-fluorenyl)AA at a level of 1.62 mmoles per kg. of diet for 8 months, had developed mammary tumors and 1 ear-duct tumor by 10 months. No other tumors were observed in the female rats tested, but about half the male animals ingesting this compound during a similar period developed liver tumors, and 1 developed a tumor of the small intestine (*7*). Eleven of 16 female rats described herein developed mammary tumors and 4 developed ear-duct tumors when fed the same chemical at a level of 1.04 mmoles per kg. of diet for a longer period (average 15 months, table 2). In addition, a variety of tumors developed at other sites. These differences could be explained by the lower dietary level of feeding, by the much longer period of ingesting the chemical, and also by the difference in strain of rat. The first tumor, a mammary tumor, was observed in our experiment after 7 months. No other mammary tumors were seen until 11 or more months. If the Buffalo-strain animals had been killed at 10 months, few tumors would have been reported. These differences indicate some of the difficulties inherent in carcinogenicity studies and serve to emphasize the necessity for long test periods when long periods ensue from the start of the experiment to the appearance of neoplasms.

The attachment of the acetylamino group to carbons 1 and 3 to form

positional isomers greatly reduces or, at position-4, eliminates carcinogenic activity (tables 3–5). In the metabolism of at least 1 of these derivatives, the 3-FAA (*23*), *o*-hydroxylation occurs, and though this hydroxy compound has not yet been tested, one would expect it to be not more but probably less carcinogenic than the 3-FAA if the same relationships hold as indicated later for 2-FAA *o*-metabolites. The further observation [Weisburger *et al.* (*38*)] that dihydroxylation takes place after the intraperitoneal administration of the 1-OH and the 3-OH-2-FAA in the rat supports the concept that hydroxylation is probably the most important initial reaction of the rat to excrete the bulk of these foreign compounds (*cf. 5, 39*). Why some compounds undergoing hydroxylation and conjugation are involved in carcinogenesis and others are not is still unknown.

1-FAA and 1-FdiAA appeared to increase slightly the incidence of pituitary tumors (table 5). The rats in these 2 tests averaged 17 to 19 months of age when the experiments were terminated. The incidence of pituitary tumors in control rats of similar age was ¾ to ¼ as high, respectively. While the carcinogenic activity of both 1-FAA and 1-FdiAA was much less than the carcinogenic activity of 2-FAA or 2-FdiAA, the 1-FdiAA seemed to be somewhat more active than 1-FAA as the 2-FdiAA was more active than 2-FAA.

Seeking an explanation for the especially high incidence of tumors of the ear duct which developed in rats after prolonged ingestion of 3-iodo-2-FAA, Dyer (*5*) examined the urine after oral administration of 3-iodo-2-FAA and discovered all the material recovered to be present as the free amine, although the rate of urinary excretion of the 3-iodo-2-FAA was similar to that of 2-FAA. 3-Iodo-2-FAA is the first compound thus far tested in this laboratory which induced a preponderance of tumors of the ear duct, and few tumors elsewhere. The presence of the iodine in the 3-position would be expected to interfere with hydroxylation, and would leave only 1 O-position free for hydroxylation. The 3-position of 2-FAA is hydroxylated to a somewhat greater extent in the rat than is the 1-position (*34*). The blocking of the usual metabolic pathway of *N*-2-FAA may also be involved in this altered carcinogenic action forcing the organism to use alternative metabolic pathways for elimination of the compound.

p-Phenylazoacetanilide and *m*-tolylazoacetanilide afford other examples of alteration of carcinogenic activity by slight structural changes in the molecule. These azo dyes have been found to require at least one *N*-methyl constituent for liver carcinogenesis (*4*). However, acetylation in place of methylation of the nitrogen of the aniline ring of either of these 2 azo dyes, although eliminating liver carcinogenesis as expected, resulted in a few tumors at other sites (table 5). The elimination of liver carcinogenesis by acetylation of these 2 azo dyes may permit the development of tumors at other sites, which have longer induction periods.

It now appears from available published results on *N*-(7-OH)-2-FAA (*40, 41*) and from tests (unpublished results, this laboratory) on the 1-, 3-, 5-, and 7-hydroxy derivatives of 2-FAA that, when ingested by the rat for at least 12 months at molecular equivalent concentrations, they

are much less carcinogenic than 2-FAA. The glucuronides and sulfates of these 4 hydroxy derivatives account for a large portion (approximately 70%) of the urinary metabolites excreted by the female Buffalo-strain rat ingesting diets containing 2-FAA (*42*). Orally administered *N*-(7-hydroxy-2-fluoren-9-C^{14}-yl) acetamide yielded radioactivity in all the tissues of rats examined (*37*). Of the urinary radioactivity two-thirds was present as glucuronic and sulfuric acid conjugates; these conjugates were mostly of the compound administered. In addition, however, some 7-amino-2-fluorenol and possibly some polyhydroxylated derivatives were formed, but only small amounts were excreted unconjugated. Substantial amounts of radioactivity were also bound to liver proteins, though no liver tumors have been found in this laboratory after rats ingested, for 12 months, the 7-hydroxy derivative. These observations are in general agreement with those of Bielschowsky (*40*) and Hoch-Ligeti (*41*). Hydroxylation and conjugation appear to be used by the rat for solubilizing, in body fluids, many foreign compounds including 2-FAA and *N*-(7-OH)-2-FAA, probably so that they can be rapidly filtered out of the blood stream by the renal tubules and excreted (*cf. 39*).

The rate of excretion of these foreign chemicals could also be a factor affecting their carcinogenicity. The more rapidly excretion occurs the less carcinogenic the chemical might be although the evidence available concerning 2-FAA *versus* 3-iodo-2-FAA (*5*) does not support such a view. Evidence possibly in favor of a relation between the rapidity of hydroxylation, conjugation, and excretion of *N*-2-FAA to carcinogenic activity has been observed in the cancer-susceptible rat compared to the non-cancer-susceptible guinea pig (*43*). Excretion occurred more rapidly in the latter species. Others, too, have noted reduced carcinogenic activity after hydroxylation of carcinogens. Thus, Miller and Miller (*7*) and Miller *et al.* (*44*) have reported that certain *o*-hydroxylated biphenylamines as well as 3-hydroxy-4-acetyl aminobiphenyl are less carcinogenic than their respective biphenylamines or acetylated amines.

Reactions of hydroxylation and conjugation are commonly used by the rat to excrete many drugs (*cf. 5, 39*); carcinogens as well as noncarcinogens need further examination to determine how these reactions are involved in carcinogenesis. The initial action of carcinogens obviously must take place during their absorption and metabolism before the elimination of the compound. This might occur either before or after it combines with some cell constituent if combination is a necessary step to tumor induction. Another possibility is that a very considerable unaccounted for portion of the urinary and fecal metabolites from *N*-2-FAA may contain as yet unidentified material with carcinogenic activity.

Still another possibility is that the enzymatic oxidative and conjugative processes and other as yet unknown reactions involve alteration of cells by chemically modifying self-duplicating templates of either genetical or enzymatic origin, as suggested by Haddow (*45*), or in their enzyme-forming systems, as proposed by Potter (*46*), to an extent that one or more characteristics or functions can be transmitted to daughter cells. Such

altered cells must have an environment favorable to their continued growth, and somewhere in subsequent cell generations they must become autonomous as in endocrine gland tumors (*47*, *48*). Many cells may be altered in variable degree, but only a few may become sufficiently independent to develop into growths which are no longer integrated in harmony with the needs of the organism and hence eventually come to be recognized as neoplasms.

Foulds (*49*, *50*) believes a sequence of steps occurs which he terms "tumor progression." According to his concept a permanent heritable qualitative change in a cell or cells may occur unpredictably in any cell capable of proliferation. A period of variable length then may ensue during which initiation of the carcinogenic process takes place. It is widely recognized that tumors develop weeks and even months after carcinogen ingestion has been stopped. To cite but one example in our experiments (expt. 274, tables 2 and 3) 7-methoxy-2-fluorenylacetamide was fed for 12 months; the surviving animals were continued on the basal diet for several months longer, and during this observation period they developed a suprisingly large number of tumors—presumably long after all traces of the ingested chemical had been excreted.

An exposure of 25 days at a dietary dose level of 0.125 percent has been reported necessary to elicit tumors (*51*) by 2-FAA. The tumors appeared many months later. The question arises as to how many cell alterations are involved and how extensive they have to be to cause such changed cells to progress into cancer. It seems reasonable to believe that continued administration of the chemical carcinogen may and probably does cause many repeated cell alterations not necessarily alike. In fact, reports indicate that alterations producing striking differences in function and behavior may be brought about in tumor cells themselves. Thus, Westfall *et al.* (*52*) report variations in arginase activities of more than 400-fold in descendants from a single mouse fibroblast after cultivation *in vitro* for extended periods. The occurrence of this greatly increased arginase activity in some cultures must have resulted from a change or alteration in some of the cells during propagation *in vitro*. Woolley (*53*) also reports that changes in resistance and susceptibility occur spontaneously and rather generally among populations of leukemic cells. He considers that the function of the antimetabolite is merely that of a selective agent causing increases in resistance and/or susceptibility gradually over a period of time. One must remember that mutagens can act in ranges of effect from minute mutations to chromosome aberrations of all kinds, from that of breakage to ploidy, and that the alteration(s) may not be detected until long after their occurrence (*54*).

Other evidence supporting alterations in cellular function in carcinogenesis is provided in the report, by Cohen (*55*), of biochemical dedifferentiation in rat liver. He noted in regenerating liver and in hepatoma a striking decrease in the ornithine transcarbamylase activity, an enzyme system important in urea synthesis and one having a characteristically high activity in fully mature rat liver, but in the enzyme

system competing for carbamyl phosphate the aspartic transcarbamylase activity increases in regenerating rat liver and in hepatoma. This latter enzyme catalyzes the reaction in which carbamyl phosphate plus aspartate form the precursor substance for orotic acid, a molecule essential for pyrimidine synthesis. The urea cycle represents a highly specialized biosynthetic mechanism and when the forces for growth as in regenerating liver and in hepatoma compete with the catabolic process of urea synthesis characteristic of the highly differentiated mature rat liver, the former appear preferentially to utilize the limited amount of carbamyl phosphate available. These few observations illustrate admirably alternative pathways in synthetic processes, as discussed by Potter (*46*, *56*).

Broadly conceived, neoplasia also resembles, in some respects, normal differentiation. Many similar mechanisms may be involved in each. For example, the choice of alternative pathways may lead either to specialization or to a gain or loss of a particular enzyme or enzyme system. The latter may be attributable to the functional loss of some particular portion of the DNA ["enzyme deletion" concept of Potter (*46*)]. Assuming that alteration in the cells during developmental processes is similar to their effect in the development of neoplasia and that it occurs in the genes themselves, when does it occur? The induction of tumors by chemicals, as mentioned previously, has a comparatively long initiation period. Assuming that the effects of genes in developmental processes are similar to their effects in cancer development, then it is possible to find an analogy between the many instances in which certain genes act in the embryonic period, long before any detectable character or function becomes apparent (*54*), and the long latent period in chemical carcinogenesis, before the appearance of neoplasia. To attack effectively the problem of mechanism(s), one must start from the obvious morphologically observable neoplasia and try to work back, step by step, through developmental defects to biochemical errors of metabolism—as Garrod (*57*) termed them—and from such hopefully less complex alterations try to discover the initial activity of the genes or enzyme-forming systems (*46*). Much pleiotrophy disappears as one approaches the initial gene activity, for many physiological as well as developmental defects can be attributable to some earlier modification of the metabolism, in early life, of enzyme inhibitors (*58*) or enzymes possessing multiple specificity. If similar metabolic modifications take place during induction of neoplasia, we would expect tumors to occur in many organs and tissues and in a great variety of ways—an experimentally established fact for chemical carcinogens.

One attack on the possible mechanism(s) of carcinogenesis involves the use of isotopically labeled molecules to follow the metabolism of chemicals of variable carcinogenic potency, their distribution, their combination with proteins or with other cell constituents, and their action on cellular material. This approach has been effectively utilized in this laboratory and is one which offers practical promise for the immediate future, and one which may ascertain some of the possible pathways, "biochemical as well as biological" (*59*), involved in tumor induction.

REFERENCES

(*1*) MORRIS, H. P.: Hepatocarcinogenesis by 2-acetylaminofluorene and related compounds including comments on dietary and other influences. J. Nat. Cancer Inst. Supp. 15: 1535–1545, 1955.

(*2*) MORRIS, H. P., DUBNIK, C. S., and JOHNSON, J. M.: Studies of the carcinogenic action in the rat of 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylaminofluorene after ingestion and after painting. J. Nat. Cancer Inst. 10: 1201–1213, 1950.

(*3*) MORRIS, H. P., and FIRMINGER, H. I.: Influence of sex and sex hormones on development of hepatomas and other hepatic lesions in strain A × C rats ingesting 2-diacetylaminofluorene. J. Nat. Cancer Inst. 16: 927–949, 1956.

(*4*) MILLER, J. A., and MILLER, E. C.: The carcinogenic aminoazo dyes. Advances Cancer Res. 1: 339–396, 1953.

(*5*) DYER, H. M.: A comparison of the patterns of excretion and the carcinogenicity in the rat of 2-acetylaminofluorene and related compounds. J. Nat. Cancer Inst. 16: 11–30, 1955.

(*6*) WEISBURGER, E. K., WEISBURGER, J. H., VELAT, C. A., and MORRIS, H. P.: The fate of the methyl group in 2-methyl-C^{14}-aminofluorene including studies of the carcinogenicity of 2-methyl- and 2-dimethylaminofluorene. J. Biol. Chem. 221: 845–854, 1956.

(*7*) MILLER, J. A., SANDIN, R. B., MILLER, E. C., and RUSCH, H. P.: The carcinogenicity of compounds related to 2-acetylaminofluorene. II. Variations in the bridges and the 2-substituent. Cancer Res. 15: 188–199, 1955.

(*8*) MILLER, E. C., and MILLER, J. A.: Biochemical investigations of hepatic carcinogenesis. J. Nat. Cancer Inst. Supp. 15: 1571–1590, 1955.

(*9*) MORRIS, H. P., VELAT, C. A., and WAGNER, B. P.: The carcinogenic action of some ingested acetylaminobiphenyls in the rat. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 36, 1955.

(*10*) ———: Carcinogenicity of some ingested acetylated mono- and diaminobiphenyl compounds in the rat. J. Nat. Cancer Inst. 18: 101–115, 1957.

(*11*) WALPOLE, A. L., WILLIAMS, M. H. C., and ROBERTS, D. C.: The carcinogenic action of 4-aminodiphenyl, and 3-2′-dimethylaminodiphenyl. Brit. J. Indust. Med. 9: 255–263, 1952.

(*12*) MORRIS, H. P., DUNN, T. B., and DUBNIK, C. S.: Two transplantable rat carcinomas induced with *N*-acetyl-2-aminofluorene. J. Nat. Cancer Inst. 9: 225–228, 1948.

(*13*) WILSON, R. H., DEEDS, F., and COX, A. J., JR.: The carcinogenic activity of 2-acetaminofluorene. III. Manner of administration, age of animals, and type of diet. Cancer Res. 7: 450–452, 1957.

(*14*) MORRIS, H. P., VELAT, C. A., and WAGNER, B. P.: Unpublished data.

(*15*) RAY, F. E., and RIEVESCHL, G., JR.: 2-Acetylfluorene. Org. Syntheses Coll. 3: 23–26, 1955.

(*16*) COURTOT, C.: Étude dans la Série du Fluorène. Ann. Chim. (10) 14: 5–146, 1930.

(*17*) WEISBURGER, E. K.: Derivatives of 2-amino-7-iodofluorene. J. Am. Chem. Soc. 72: 1758–1759, 1950.

(*18*) WEISBURGER, E. K., WEISBURGER, J. H., and RAY, F. E.: The mercuration of 2-aminofluorene. J. Org. Chem. 16: 1697–1700, 1951.

(*19*) SAWICKI, E., and RAY, F. E.: *N*-trifluoroacetyl derivatives of carcinogenic amines. J. Am. Chem. Soc. 75: 2226–2267, 1953.

(*20*) ———: *N*-fluoroacetyl derivatives of carcinogenic amines. J. Am. Chem. Soc. 75: 4346, 1953.

(*21*) WEISBURGER, J. H., WEISBURGER, E. K., and MORRIS, H. P.: Analogs of the carcinogen 2-acetylaminofluorene: the isomeric 4-acetylaminofluorene. J. Am. Chem. Soc. 74: 4540–4543, 1952.

(*22*) WEISBURGER, E. K., and WEISBURGER, J. H.: An improved synthesis of 1-aminofluorene. J. Org. Chem. 18: 864–867, 1953.

(23) WEISBURGER, E. K.: 2-Hydroxy-3-acetylaminofluorene, a metabolite of 3-acetylaminofluorene in the rat. J. Am. Chem. Soc. 77: 1914–1915, 1955.

(24) DIELS, O.: Zur kenntniss de fluorens. (I. Mittheilung.) Ber. dtsch. chem. Ges. 34: 1758–1768, 1901.

(25) WEISBURGER, J. H., and WEISBURGER, E. K.: 2-Acetamido-7-methoxyfluorene. J. Chem. Soc. 758–759, 1954.

(26) CAMPBELL, N., ANDERSON, W., and GILMORE, J.: The structure of aromatic compounds. Part II. J. Chem. Soc. 1: 446–451, 1940.

(27) SCHULMAN, S.: Fluorene derivatives for cancer research. J. Org. Chem. 14: 382–387, 1949.

(28) FOURNEAU, E., TRÉFOUËL, J., NITTI, F., BOVET, D., and TRÉFOUËL, M^Me.: Action antistreptocorcique des derives sulfaures organiques. Compt. rend. Acad. Sc. 204: 1763–1766, 1937.

(29) HIRS, C. H. W.: Two new derivatives of 2-aminofluorene. J. Am. Chem. Soc. 71: 1893, 1949.

(30) CHAPLIN, H. O., and HUNTER, L.: The associating effect of the hydrogen atom. Part II. Substituted anilides and related substances. J. Am. Chem. Soc. 1: 375–382, 1938.

(31) BIELSCHOWSKY, F.: Carcinogenic action of 2-acetyl-aminofluorene and related compounds. Brit. M. Bull. 4: 382–385, 1947.

(32) SPJUT, H. J., and EICHWALD, E. J.: Induction of mammary tumors by injection of *N*-2-fluorenylacetamide into artificial cecal pouches in rats. J. Nat. Cancer Inst. 18: 473–479, 1957.

(33) HADDOW, A., SCOTT, C. M., and SCOTT, J. D.: The influence of certain carcinogenic hydrocarbons on body growth in the rat. Proc. Roy. Soc., London, ser. B 122: 477–507, 1937.

(34) WEISBURGER, J. H., WEISBURGER, E. K., and MORRIS, H. P.: Urinary metabolites of the carcinogen *N*-2-fluorenylacetamide. J. Nat. Cancer Inst. 17: 345–361, 1956.

(35) RAY, F. E., and ARGUS, M. F.: Studies on the metabolism, distribution, and excretion of 2-*p*-toluenesulfonamidofluorene-S^{35} in the rat. Cancer Res. 11: 783–787, 1951.

(36) WEISBURGER, J. H., WEISBURGER, E. K., and MORRIS, H. P.: The effect of riboflavine on the metabolism of 2-acetylaminofluorene-9-C^{14} in rats. J. Nat. Cancer Inst. 13: 37–47, 1954.

(37) ———: The biosynthesis of *N*-(7-hydroxy-2-fluoren-9-C^{14}-yl)-acetamide from *N*-2-fluoren-9-C^{14}ylacetamide, and its metabolism in the rat and in the guinea pig. Arch. Biochem. 80: 187–198, 1959.

(38) WEISBURGER, J. H., WEISBURGER, E. K., GRANTHAM, P. H., and MORRIS, H. P.: The biosynthetic preparation of C^{14}-labeled *N*-(1- and 3-hydroxy-2-fluorenyl)-acetamides and their metabolism in rats and guinea pigs. J. Nat. Cancer Inst. 22: 825–844, 1959.

(39) BRODIE, B. B., MAICKEL, R. P., and JONDORF, W. R.: Termination of drug action by enzymatic inactivation. Fed. Proc. 17: 1163–1174, 1958.

(40) BIELSCHOWSKY, F.: A metabolite of 2-acetamidofluorene. Biochem. J. 39: 287–289, 1945.

(41) HOCH-LIGETI, C.: Effect of feeding 7OH-2-acetaminofluorene to albino rats. Brit. J. Cancer 1: 391–396, 1947.

(42) WEISBURGER, E. K., and WEISBURGER, J. H.: Chemistry, carcinogenicity, and metabolism of 2-fluorenamine and related compounds. Advances Cancer Res. 5: 331–431, 1958.

(43) WEISBURGER, J. H., WEISBURGER, E. K., and MORRIS, H. P.: Differences in metabolism of *N*-2-fluorenylacetamide in the guinea pig and the rat. Cancer Res. 18: 1039–1047, 1958.

(44) MILLER, E. C., SANDIN, R. B., MILLER, J. A., and RUSCH, H. P.: The carcinogenicity of compounds related to 2-acetylaminofluorene. III. Aminobiphenyl and benzidine derivatives. Cancer Res. 16: 525–534, 1956.

(*45*) HADDOW, A.: Chemical carcinogens and their modes of action. Brit. M. Bull. 14: 79–92, 1958.

(*46*) POTTER, V. R.: The biochemical approach to the cancer problem. Fed. Proc. 17: 691–697, 1958.

(*47*) MORRIS, H. P.: The experimental development and metabolism of thyroid gland tumors. Cancer Res. 3: 51–115, 1955.

(*48*) FURTH, J.: Discussion of problems related to hormonal factors in initiating and maintaining tumor growth. Cancer Res. 17: 454–463, 1957.

(*49*) FOULDS, L.: Tumor progression. Cancer Res. 17: 355–356, 1957.

(*50*) ———: Neoplastic development. *In* The Chemical Basis of Development. Baltimore, The Johns Hopkins Press, 1958, pp. 680–703.

(*51*) WILSON, R. H., DEEDS, F., and COX, A. J., JR.: The carcinogenic activity of 2-acetaminofluorene. II. Effect of concentration and duration of exposure. Cancer Res. 7: 444–449, 1947.

(*52*) WESTFALL, B. B., PEPPERS, E. V., EVANS, V. J., SANFORD, K. K., HAWKINS, N. M., FIORAMONTI, M. C., KERR, H. A., HOBBS, G. L., and EARLE, W. R.: The arginase and rhodanese activities of certain cell strains after long cultivation *in vitro*. J. Biophys. & Biochem. Cytol. 4: 567–570, 1958.

(*53*) WOOLLEY, G. W.: Tumor cell resistance to antimetabolites and its possible genetic implications. *In* 6th Ann. Symp. in Cancer Res. M.D. Anderson Hospital and Tumor Clinic. Feb. 26–28, 1959.

(*54*) HADORN, E.: Role of genes in development processes. *In* Chemical Basis of Development. Baltimore, The Johns Hopkins Press, 1958, pp. 779–793.

(*55*) COHEN, P. P.: Discussion of neoplastic development. *In* The Chemical Basis of Development. Baltimore, The Johns Hopkins Press, 1958, pp. 702–703.

(*56*) POTTER, V. R.: Discussion of neoplastic development. *In* The Chemical Basis of Development. Baltimore, The Johns Hopkins Press, 1958, pp. 700–703.

(*57*) GARROD, A. E.: Inborn errors of metabolism. Frowde, London, 1923.

(*58*) FOUTS, J. R., and ADAMSON, R. H.: Drug metabolism in the newborn rabbit. Science 129: 897–898, 1959.

(*59*) BERENBLUM, I.: The mechanisms of carcinogenesis: The role of carcinogenic metabolites. Acta Unio internat. contra cancrum 15: 22–30, 1959.

A Study of Urinary β-Glucuronidase Activity in the Production of Bladder Tumors in Rats [1,2]

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SUMMARY

Bladder tumors developed in female Fischer-strain but not in female Buffalo-strain rats fed diets containing *N*-2-fluorenylacetamide and 2 percent DL-tryptophan or 0.8 percent indole. Only insignificant differences between rats of the two strains were observed in the weight curves, in urinary β-glucuronidase activities, or in chromatograms of aromatic metabolites of tryptophan and indole in the urine. Little evidence was obtained to support the hypothesis that urinary β-glucuronidase and *o*-aminohydroxy metabolites of tryptophan were involved. The increase in the number and amounts of aromatic metabolites excreted in the urine by the rats fed tryptophan or indole suggests the possibility that these metabolites may affect the bladder epithelium as a contributing factor in the induction of cancer by *N*-2-fluorenylacetamide.—J. Nat. Cancer Inst. 24: 181–196, 1960.

THE POSSIBILITY that urinary β-glucuronidase might be involved in the induction of bladder cancer by hydrolyzing glucuronides of *o*-aminohydroxy metabolites of tryptophan has been suggested by Boyland and associates (*1*). Since indole- or DL-tryptophan-supplemented diets containing 0.06 percent *N*-2-fluorenylacetamide (2-FAA), but not 2-FAA alone, have been observed by Dunning, Curtis, and Maun (*2, 3*) to induce bladder tumors in rats, it seemed important for a better understanding of this problem to investigate the effects of these supplements on the urinary glucuronidase activity of rats.

Analyses were made of the urine of female Buffalo-strain rats fed diets similar to those used by Dunning *et al.* (*2, 3*). Bladder tumors failed to develop in the rats, but they died in 7 to 12 months after the development of mammary cancers. Cancers of other sites occurred, including tumors of the ear, the liver, the harderian gland, and several miscellaneous sites. Lest the failure of bladder tumors to develop in the Buffalo rat should be the result of a strain resistance and/or a difference that involves

[1] Received for publication May 14, 1959.

[2] Presented in part at the annual meeting of the American Association for Cancer Research, Philadelphia, Penna., April 1958. (Abstract of paper, Proc. Am. Assoc. Cancer Res. 2: 293, 1958.)

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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the metabolism of the indolic compounds, the study was repeated in part with female rats of the Fischer line 344.[4] This strain has been shown to be susceptible to the induction of bladder tumors (*2, 3*).

In this experiment, 7 of 11 Fischer rats fed the DL-tryptophan-supplemented diet died in 1 to 5.25 months, with no visible tumors. Nodules were seen in the urinary bladders of 4 rats autopsied after 8 to 11.5 (average 10) months on the diet. Histologic examination of these rats revealed papillomatous tumors of the bladder.[5] Seven of 11 Fischer rats fed the diet containing indole died after 2 to 5.5 months. Of 4 that survived for 9.5 to 11.75 (average 10.4) months, 1 had a nodular sarcoma of the kidney, 1 had papillomatous hyperplasia of the bladder, and 2 had bladder nodules diagnosed as papillomatous tumors. In contrast, 5 female Buffalo-strain rats that survived from 8.5 to 11 (average 9.3) months on the tryptophan-supplemented diet and 4 that survived 8 to 12 (average 9.8) months on the 2-FAA diet containing indole failed to develop bladder tumors. Repeated studies have shown that tumors of the bladder rarely appear in the female Buffalo-strain rat fed 2-FAA diets containing either added indole or tryptophan.

The findings reported here indicate insignificant differences between Buffalo- and Fischer-strain female rats, in urinary β-glucuronidase activity and the patterns of urinary tryptophan metabolites. The findings also provide little evidence that the induction of bladder tumors in rats fed fluorenylacetamide diets, supplemented with DL-tryptophan or indole, could be due to the liberation in the bladder of free *o*-phenolic metabolites of tryptophan from glucuronides by the urinary enzyme.

MATERIALS AND METHODS

Animals, diets, and general procedures.—Female Buffalo-strain rats 3 months of age were divided into 6 groups of 5 each, except for the control group on the basal diet, in which there were 8 rats. The different groups were then fed the diets seen in table 1. Several months later, for further comparison of the effects upon urinary glucuronidase activity, 6 groups of 5 rats each, 2 or 3 months of age, were fed the same basal diet, F, supplemented with 1 percent L-tryptophan, 2 percent L-tryptophan, and 1 percent D-tryptophan, and diets with these same tryptophan supplements in addition to 0.06 percent 2-FAA.

The Fischer-strain rats were placed on the experimental diets at 4 months of age. Analyses were made of the urine of 8 Fischer rats fed the control diet, 5 fed the 2-FAA diet supplemented with 2 percent DL-tryptophan, and 5 fed the 0.8 percent indole diet containing the carcinogen.

The animals were kept in individual metal cages with raised wire-cloth bottoms. Food and water were provided *ad libitum*. Body weights and food consumption were recorded weekly. During the experimental period,

[4] The original source of the NIH Fischer-strain rats was the laboratory of Dunning and Curtis, in 1949.
[5] The results of the carcinogenic studies will be reported in detail in a separate paper.

TABLE 1.—Composition of diets

Ingredients	Basal diet F* (gm.)
Vitamin-free casein	260.0
Salt mixture (185a)	40.0
Cellu flour vitamin mix†	20.0
Dextrin	530.0
Lard + α-tocopherol ‡	10.0
Lard	140.0
Cod-liver oil	4.0
Choline chloride	2.0

*Other diets contained the basal diet supplemented as follows: F + 0.06% *N*-2-fluorenylacetamide (2-FAA); F + 2% DL-tryptophan replacing an equal percentage of casein; F + 2% DL-tryptophan and 0.06% 2-FAA; F + 0.8% indole replacing 1% of casein; F + 0.8% indole and 0.06% 2-FAA.

† 20 gm. Cellu flour contains: B_1 · HCl, 8 mg.; B_2, 16 mg.; B_6 · HCl, 2 mg.; niacin, 4 mg.; and calcium pantothenate, 40 mg.

‡ 150 mg. α-tocopherol per kg. of diet.

at intervals between 3 and 18 weeks, pairs of rats were transferred to individual all-glass metabolism cages that were designed for separation of urine and feces. Four rats were used during each collection period and selected so that comparisons could be made on the same day (1) between rats fed the control diets with and without the fluorenylacetamide supplement; (2) between rats fed the unsupplemented basal diet F and each of the tryptophan- and indole-supplemented diets without the carcinogen; and (3) between rats fed the tryptophan- and indole-supplemented diets containing the carcinogen. The urine was collected in iced tubes in Dewar flasks for periods of 24 hours, at which time the rats were made to micturate by pressure to empty the bladder. The cages were then washed sparingly. Food was withheld during the collection period to avoid contamination of the urine by the supplements. Water was available at all times.

Since consistent ingestion of even restricted amounts of food was not obtained when the rats were placed in the metabolism cages for 24-hour periods, it was decided to withhold food during the collection period. After much of the study was completed a final comparison by chromatography of urinary metabolites was undertaken, using forced feeding of aqueous suspensions of diets F + 2 percent DL-T + 2-FAA and F + 0.8 percent indole + 2-FAA to insure the presence of large amounts of metabolites. The suspension consisted of a ground mixture of 1 part food to 1.5 parts water; 4 ml. was administered by stomach tube at the beginning, and 4 ml. at the 6th hour of the 24-hour collection period. With this procedure more β-glucuronidase activity was noted in the urine than that noted in the previous experiments when food had been withheld. It seemed desirable therefore to compare the urinary glucuronidase activities of a series of rats, with food withheld and after forced feeding of some of the diets, and to re-examine, by chromatography, urine from force-fed rats. For this purpose groups each of 4 Buffalo- and 4 Fischer-strain female rats, 3.75 months of age, were maintained on diets F, F + 2-FAA, F + 2 percent DL-T + 2-FAA, and F + 0.8 percent indole + 2-FAA. The rats were used in paired groups of 4 rats, one of each

pair force-fed, the other with food withheld. A week later each experiment was repeated, reversing the force-fed and fasted rats.

With distilled water the urine from each rat was made to the same volume, usually 15 ml.; analyses in duplicate were begun at once. Total nitrogen was determined by a micro-Kjeldahl method, and β-glucuronidase by the method of Talalay, Fishman, and Huggins (*4*), with phenolphthalein glucuronidate as the substrate, incubated at 38° C. for 1 hour at *p*H 4.5.

Paper chromatography patterned after the procedure of Dalgliesh (*5*, *6*) was used to identify urinary tryptophan and indole metabolites. An aliquot of urine was adjusted to *p*H 4 with glacial acetic acid, and was treated with "deactivated" carbon. The water-washed carbon residue was eluted with 8:100 aqueous dilution of phenol liquefactum B.P., and again washed with water. The combined phenol eluate and wash were distilled to dryness *in vacuo* in a Flash-Evaporator. The final water-washed residue was taken up in 0.25 ml. H_2O and used in amounts of 10 to 30 μl. in ascending two-dimensional chromatography on Whatman #1 paper cylinders, 9 × 7.5 inches, which is a modification of the Dalgleish procedure. The first solvent phase consisted of *n*-butyl alcohol: glacial acetic acid:H_2O (4:1:5 by volume). After drying in a hood overnight, the cylinders were remade by 90° rotation and placed in the second solvent phase of 20 percent aqueous KCl. For urine from rats on each diet, two-dimensional descending chromatography was also used with 10 to 50 μl. of the material on sheets of Whatman #1 paper, 22 × 18 inches, according to the Dalgliesh method (*6*). For each diet also, a second elution was made of the aqueous-phenol-eluted carbon with NH_4OH:8 to 100 aqueous phenol (1:5 by volume). The ammonia phenol eluates were chromatographed separately. After drying, the chromatograms were examined for fluorescence under an ultraviolet lamp[6] and then sprayed with Ekman (*7*) or Ehrlich (*5*) reagents. Pooled urine from 2 rats and later from 3 rats, on the same diet, was divided into equal aliquots to compare (1) freshly collected 24-hour urine; (2) the same urine after incubation at 38° C. for 20 hours, "auto digestion"; and (3) the urine after incubation for 20 hours after addition of bacterial glucuronidase. In determining the effect of "auto digestion," comparisons were made at various times with (1) the addition of one-third volume of 0.15 M acetate buffer at *p*H 6; (2) a preliminary ether extraction which was found to result in maintenance of the original *p*H of the urine, and (3) the addition of one-third volume of acetate buffer, *p*H 4.5, after adjustment of the urine to *p*H 4.5 with acetic acid, and with a layer of toluene. For bacterial glucuronidase digestion, 3 mg. of the dry preparation (Sigma) was added per ml. of diluted urine, and one-third volume of acetate buffer at *p*H 6.0.

Since no *conjugated* metabolites of tryptophan were available as standards, a preliminary study was made of the urine of rats after administration, by stomach tube, of aqueous suspensions of anthranilic acid,

[6] Blak-Ray longwave ultraviolet, Ultra Violet Products, Inc., San Gabriel, Calif.

DL-kynurenine, and the urine of rats fed a B_6-deficient diet, after administration of a challenging dose of L-tryptophan. Chromatograms of aliquots of phenol eluates of each carbon-treated urine, before and after incubation of the urine with bacterial β-glucuronidase, led to the identification of 2 conjugated metabolites as glucuronides, anthranilic acid glucuronide and "compound B," which Dalgliesh (*6*) considered a conjugate of xanthurenic or kynurenic acid. These were the only metabolites that disappeared when the urine was incubated with bacterial β-glucuronidase. By comparison with synthetic compounds and/or the "map of spots," and other information published by Dalgliesh (*5*, *6*), xanthurenic acid, kynurenic acid, L- and D-kynurenine, 3-hydroxykynurenine, N^{α}-acetylkynurenine, N^{α}-acetyl-3-hydroxykynurenine, 3-hydroxykynurenine-O-sulfate, "compound C," and possibly a trace of 3-hydroxyacetophenone were identified in addition to anthranilic acid. Anthranilic acid was the only compound that increased in amount as a result of incubation of the urine with bacterial β-glucuronidase.

RESULTS

Total Urinary Nitrogen

The total nitrogen contents of the 24-hour samples of urine were relatively constant, increasing a little as the rats increased in weight and consumed more food. Throughout the experimental period the urine of rats fed the 2-FAA diets contained less nitrogen than that of rats of the same age, but maintained, before the collection period, on the corresponding control diets without the carcinogen. When forced feeding was used, the amount of food administered was less than half that generally ingested in 24 hours when the rat was fed *ad libitum* outside the metabolism cage. The nitrogen content of the 24-hour sample of urine from the force-fed rat averaged 127 to 153 percent that from the same rat with food withheld during the collection period.

Urinary β-Glucuronidase—Food Withheld

Table 2 demonstrates the large variation in β-glucuronidase activity found in 24-hour samples of urine of rats of the same age and maintained on the same diets under identical experimental conditions, with food withheld for 24 hours during the period of collection beginning in the morning. Frequently wide variations in enzyme activity were found in the same rats during different experimental periods. When the glucuronidase activities are expressed on the basis of units per mg. of urinary nitrogen, the relative amounts of activity from rats on the different diets are the same as when the total activities are compared.

Urinary β-Glucuronidase—Force Fed

Table 3 shows a comparison of glucuronidase activity in the urine of individual rats, with forced feeding and with food withheld during the

collection periods. The variation in urinary β-glucuronidase activity observed in different rats and even in the same rats under identical experimental conditions emphasizes the limitations in the use of average values and in generalizations. It is apparent that, with the possible exception of the Fischer rats fed the indole-2-FAA diet, the total glucuronidase activity of the urine of the rat, when force fed, is usually, but not always, greater than when food is withheld from the same rat during the collection period. When the activity is expressed as units per mg. of urinary nitrogen, however, there is little evidence of increased activity as a result of forced feeding of the basal diet and the basal diet containing 2-FAA. With the diet containing 2-FAA and 2 percent tryptophan, however,

TABLE 2.—β-Glucuronidase activity in urine* of rats on various diets

Diet†	Mean, ±S.E., and range in activity as total units excreted in 24 hours‡	
	Buffalo ♀	Fischer ♀
F	369 ± 68 (84–1050)	840 ± 134 (135–1987)
F + 2-FAA	973 ± 268 (456–1805)	
F + 2% DL-T	1779 ± 600 (448–3450)	
F + 2% DL-T + 2-FAA	602 ± 182 (98–1560)	418 ± 120 (0–957)
F + 0.8% indole	458 ± 137 (15–1110)	
F + 0.8% indole + 2-FAA	537 ± 125 (319–1188)	489 ± 63 (210–717)
F + 1% L-T	723 ± 172 (324–1356)	
F + 1% L-T + 2-FAA	1287 ± 587 (348–3872)	
F + 1% D-T	653 ± 191 (240–1005)	
F + 1% D-T + 2-FAA	85 ± 65 (15–276)	
F + 2% L-T	322 ± 40 (240–400)	
F + 2% L-T + 2-FAA	568 ± 253 (248–1260)	

*Urine collected for periods of 23 hours while *food was withheld*.

†2-FAA = 0.06% *N*-2-fluorenylacetamide in diet; T = tryptophan.

‡Average of all analyses ± standard errors (*8*) and range (in parentheses) during 3d through 18th week of feeding. One unit of β-glucuronidase activity is the amount of enzyme that liberates 1 μg. of phenolphthalein in 1 hour at 38° C. from phenolphthalein glucuronide at *p*H 4.5 under standard conditions (*4*).

there was considerably more β-glucuronidase activity per mg. urinary nitrogen with forced feeding, in 6 of 9 comparisons with Buffalo rats and in 9 of 10 comparisons with rats of the Fischer strain.

Table 4 contains average values for the percentage of activity in individual rats when the various diets were force fed compared with the total activity in the urine of the same rat when food was withheld. Although the increase in urinary β-glucuronidase activity with forced feeding compared with food withheld was greatest when the diet contained 2 percent DL-tryptophan in addition to *N*-2-fluorenylacetamide, the values for total activity in these rats was no greater than that in the rats force fed the 2-FAA diet without the tryptophan supplement.

TABLE 3.—Comparison of urinary β-glucuronidase activity of rats, with food withheld and with forced feeding*

TABLE 3.—Comparison of urinary β-glucuronidase activity of rats, with food withheld and with forced feeding*

Diet†	Number of Buffalo ♀ rats	Units of β-glucuronidase‡				Number of Fischer ♀ rats	Units of β-glucuronidase‡			
		Food withheld		Force fed			Food withheld		Force fed	
		Total	Total / mg. N	Total	Total / mg. N		Total	Total / mg. N	Total	Total / mg. N
F (basal)	7000			735	4	6992			3275	23
	"	735	8	1192	7	"	2292	27	3144	20
	7001	256	2			6993			567	4
	"	990	8	1330	8	"	208	2	741	5
	7002	1776	13	4464	27	6994	297	3	1386	10
	7003	147	14	553	3	6995	1044	9	1068	8
						"	2976	27	2838	17
F + 2-FAA	7004	1308	12	1706	11	6996	628	4	dead	
	7005	2250	14	3360	21	6997	1584	13	2271	17
	7006	783	9	1347	9	6998	951	10	1497	10
	7007	1308	20	2184	18	6999	1005	9	4968	33
F + 2% DL-T + 2-FAA	7008	276	2	1860	10	6746	381	5	2592	16
	"	639	9	1008	5	"	385	3	1341	11
	"			580	3	"	501	6		
	7009	480	7	3000	23	6749	186	1	1872	12
	"	974	10	639	5	"	327	4	2856	17
	"			2736	13	"	2100	18		
	7010	600	7	1296	10	6751	537	5	2403	16
	7011	1455	9	3321	23	"	352	4	1863	11
	"	325	3	1749	12	6752	733	9	2460	17
F + 0.8% indole + 2-FAA	7012	237	2			6757	625	5		
	"	576	6	731	5	"	511	7	631	4
						"	447	5	690	5
	7013			579	4	6758			445	3
	"	743	8	438	4	"	450	5	1683	12
						6760	1530	15		
						"	432	5	1710	12
	7014	1143	9	5496	28	"	246	2	139	1
	7015	1800	15	4296	25	6761	266	3	267	2
	"			3744	25	"	541	5	774	5

*Urine collected for 24-hour periods at weekly intervals; one time with food withheld; the next week 3.2 gm. of food in 8 ml. of aqueous suspension administered by stomach tube in 2 equal volumes, at beginning and at 6th hour of collection period.

†Rats fed *ad libitum* except as mentioned in footnote *; 2-FAA = 0.06% *N*-2-fluorenylacetamide; T = tryptophan.

‡ $\frac{\text{Total}}{\text{mg. N}}$ = total units per mg. urinary nitrogen.

TABLE 4.—Average percentage urinary β-glucuronidase activity of individual rats when force fed compared with the activity when food was withheld*

Diet	Buffalo ♀ rats		Fischer ♀ rats	
	Total units	Units/mg. N	Total units	Units/mg. N
	Percent	Percent	Percent	Percent
F	257 ± 70 S.E.	144 ± 63 S.E.	209 ± 48 S.E.	146 ± 34 S.E.
F + 2-FAA	154 ± 10 S.E.	108 ± 15 S.E.	264 ± 117 S.E.	199 ± 89 S.E.
F + 2% DL-T + 2-FAA	347 ± 56 S.E.	222 ± 43 S.E.	552 ± 85 S.E.	416 ± 96 S.E.
F + 0.8% indole + 2-FAA	214 ± 60 S.E.	154 ± 37 S.E.	174 ± 38 S.E.	119 ± 33 S.E.

* Percentage: β-glucuronidase activities of $\frac{\text{force fed}}{\text{food withheld}} \times 100$ of averages of all comparative values for each individual rat.

Chromatographic Studies of Urinary Metabolites of Tryptophan and Indole

Few and relatively small amounts of tryptophan metabolites were found in paper chromatograms of the urine of rats fed the basal diet or this diet containing *N*-2-fluorenylacetamide (*9*). When rats were fed the indole or the DL-tryptophan diets with or without 2-FAA, the urine contained a greater number and relatively large amounts of detectable aromatic metabolites. Seventy-five different fluorescent and/or staining spots were seen in the chromatograms. Not more than 25 of the 75 were observed in chromatograms of the urine of rats fed diets that did not contain indole or added DL-tryptophan. Many of these materials were seen in comparatively small amounts and some of them appeared only after incubation of the urine with bacterial β-glucuronidase.

With the indole and DL-tryptophan diets significant increases were observed in the amount of anthranilic acid glucuronide compared with that in the urine of rats on diets that did not contain these supplements. Anthranilic acid glucuronide was sometimes noted in one large spot that showed constriction in the center. Chromatograms of urine from rats on diets F + 2-FAA, F + indole, and F + indole + 2-FAA showed 2 distinct spots with the same brilliant violet fluorescence and the same colors when stained for identification. Both spots proved to be glucuronides. This led us to suspect that anthranilic acid glucuronide and a glucuronide of a derivative of anthranilic acid were probably present.

"Compound B," a glucuronide, and "compound C" were suspected, by Dalgliesh (*6*), of being conjugates of xanthurenic or kynurenic acids. "B" was found in chromatograms of the urine from rats fed any one of the diets, but was observed in greatest amounts in urine from animals fed DL-tryptophan, with and without 2-FAA. Rats on the indole diet containing 2-FAA excreted more of "compound B" than those on the control indole diet. "Compound C" was observed in largest amounts in the urine of rats fed the DL-tryptophan supplement and 2-FAA. It was not found when the diet contained 2-FAA only, or 2-FAA and indole.

The rats fed the DL-tryptophan-supplemented diets excreted much greater amounts of kynurenic and xanthurenic acids than those fed the indole-supplemented diets, and in addition, the urine contained tryptophan, D-kynurenine, possibly traces of N^{α}-acetylkynurenine, 3-OH-kynurenine-O-sulfate, 2-amino-3-hydroxyacetophenone, and its O-sulfate. *o*-Aminohippuric acid was observed in the urine of Buffalo rats maintained on and force fed diets of F + DL-T, F + indole, and F + indole + 2-FAA, but not in that of Fischer rats fed F + indole + 2-FAA. The metabolites observed in largest amounts in the urine of rats fed the indole diet, with and without 2-FAA, were indican, another compound that is suspected of being indoxyl glucuronide, which is an unidentified glucuronide with a rose-flame fluorescence, in the chromatographic neighborhood of indican, and 3 unidentified metabolites that move little, if any, in the aqueous KCl phase. Two of these materials stained the paper a bright navy blue similar to the color of the urine-stained hair of rats fed indole. Of these

TABLE 5.—Urinary metabolites influenced by incubation with bacterial β-glucuronidase*

Number of compound and chromatographic identity†‡	Diet§											
	F + 0.8% indole			F + 0.8% indole + 2-FAA			F + 2% DL-T			F + 2% DL-T + 2-FAA		
	Before	*"Auto"*	*Bact. β-g.*	*Before*	*"Auto"*	*Bact. β-g.*	*Before*	*"Auto"*	*Bact. β-g.*	*Before*	*"Auto"*	*Bact. β-g.*
1. Anthranilic acid glucuronide‖	+++	+++	±	++++	+++	±	++	+	(−)	++++	+++	±
2. Anthranilic acid¶	(−)	(−)	+	(−)	++	+++	(−)	± ?	(−)	(−)	+ ?	+ ?
3. "Compound B"**	+±	(−)	(−)	++	++	(−)	++	+	(−)	+++	++±	(−)
4. "Indoxylglucuronide" ††	++	++	(−)	++++	++++	(−)	(−)	(−)	(−)	(−)	(−)	(−)
5. R_F P 56–51 Ek. (−); KCl 56–33, rose-flame fl., Ehr. (−)	+±	+±	(−)	++++	++++	(−)	(−)	(−)	(−)	(−)	(−)	(−)
5a. R_F P 71–62 Ek. (−); KCl 7–2, rose-flame fl., Ehr. (−)	(−)	(−)	+±	(−)	(−)	+++	(−)	(−)	(−)	(−)	(−)	(−)
6. R_F P 89–66 Ek. (−); KCl 8–0.6 gray-white fl., Ehr. (−)	+++	++	(−)	++++	+++	+	(−)	(−)	(−)	(−)	(−)	(−)

*Incubation at 38° C. for 20 hrs.: *Before:* no incubation; *"Auto":* incubation without added glucuronidase, *p*H 4.5; *Bact. β-g.:* incubated after addition of bacterial β-glucuronidase, *p*H 6.

†Small amounts of additional unidentified compounds appearing or disappearing during incubation are mentioned in text.

‡Paper chromatography: Whatman paper #1, 22.5" × 18"; solvent #1—P = Partridge (*n*-butyl alcohol:glacial acetic acid:water) (4:1:5 by vol.); solvent #2—20 percent aqueous KCl, $R_F \times 100$; Ehr. = Ehrlich stain; Ek. = Ekman stain; fl. = fluorescence.

§Rats maintained on same diets that were force fed: F is basal diet.

‖With diets containing indole there were 2 distinct spots one above the other, in Partridge solvent, with approximately the same R_F in KCl, and showing properties of anthranilic acid glucuronides; with all other diets one elongated spot was present, frequently showing a constriction in center.

¶Anthranilic acid sometimes showed an atypical color in fluorescence. It was present in very small amounts compared with the anthranilic acid glucuronide that disappeared.

**Suggested by Dalgliesh (*6*), as a conjugate of xanthurenic or kynurenic acid.

††Tentatively assumed to be indoxylglucuronide.

metabolites appearing in the urine of indole-fed rats, indican, only, in relatively small amounts, was found in the urine of rats not fed indole diets. Indole was not found in the chromatograms, but we have not succeeded in recovering added indole, by the procedure used.

Table 5 lists substances that were observed in the urine of rats on one or more of the experimental diets and that were affected by incubation of the urine after the addition of bacterial β-glucuronidase. It is apparent from the table that there were more glucuronides detectable in the phenol eluates of the carbon from urine of rats fed the indole diet than from those fed the DL-tryptophan-supplemented diet. With both diets, urine that was incubated with bacterial β-glucuronidase showed the absence of or a significant decrease in anthranilic acid glucuronide and "compound B," and the appearance, sometimes, especially after the indole diets, of a very small amount of anthranilic acid. The constriction in the spot, if just one was present, and the appearance in some of the chromatograms of 2 contiguous spots that showed the same fluorescence and staining behavior and disappeared upon incubation with bacterial β-glucuronidase (*footnote* ‖, table 5) suggests the possibility of the presence of 2 glucuronides. It is possible that the anthranilic acid glucuronide spot contains also, or is adjacent to, the glucuronide of 3-hydroxyanthranilic acid. No free 3-hydroxyanthranilic acid (3-OH-AA) was observed. However, with the same chromatographic procedure, 20 μg. of standard 3-OH-AA showed instability in recovery experiments yielding always a small spot characteristic of 3-OH-AA and one unidentified breakdown product. It is probable that a very small amount of free 3-OH-AA in the urine would not be recovered, and that the failure to find more free anthranilic acid after incubation of the urine with bacterial β-glucuronidase and disappearance of the large amount of glucuronide could conceivably be due to the presence of some 3-OH-AA glucuronide and the instability of the free 3-OH-AA that would be liberated during incubation.

In addition to the 2 conjugates mentioned previously, the urine of DL-tryptophan-fed rats contained one other compound, and that of indole-fed rats contained 5 other materials that disappeared during incubation with the bacterial enzyme. A compound, #5*a*, that fluoresced like #5 (*see* table 5), was observed in chromatograms of the bacterial glucuronidase-digested urine from the indole-fed rats. It moved further in the organic solvent mixture and very little in KCl, which suggested that it might have arisen from hydrolysis of the glucuronide #5.

Chromatograms of the bacterial β-glucuronidase-digested urine from rats fed either indole- or DL-tryptophan-supplemented diets showed small amounts of 7 and 5 unidentified fluorescing materials, respectively, which were not present in the urine before treatment. Two of these compounds were also seen in urine that had been incubated without addition of the bacterial enzyme. None of these materials was stained by Ekman or Ehrlich reagents. Only one of them appeared to be common to the urine of rats fed diets containing the 2 different supplements, indole and DL-tryptophan.

A compound from the urine of rats fed the indole diet had the same R_F as 3-hydroxykynurenine-O-sulfate, but had a pink fluorescence and did not stain with Ekman or Ehrlich reagents. It also disappeared when the urine was incubated at a buffered *p*H of 4.5, without added enzyme. Incubation of the urine of rats fed any one of the diets resulted in the disappearance of small amounts of anthranilic acid glucuronide and of "compound B," if the urine was first buffered to *p*H 4.5. Sometimes a small amount of anthranilic acid appeared as a result of this "auto" digestion at *p*H 4.5. When large amounts of these 2 glucuronides were present, however, it was difficult to determine whether any had been hydrolyzed, a finding in striking contrast to the effect of incubation with bacterial glucuronidase, when all but traces of the glucuronides disappeared. There was no evidence of hydrolysis of any conjugates of metabolites of tryptophan or indole when the urine was incubated for 20 hours at a buffered *p*H of 6, or at the *p*H of the urine, 5.9–6.2, maintained by a preliminary ether extraction.

DISCUSSION

The source of urinary β-glucuronidase is not known, but Boyland *et al.* (*1*) reported experimental evidence that it is probably derived from the circulating blood. The source then could be the liver or any other organ, as well as the bladder epithelium. Variation in activity in the urine of different rats and of individual rats during different collection periods would seem to reflect variation in the amount of the enzyme present in some tissue or tissues. Tumor tissue which has been observed to have high β-glucuronidase activity (*10*) was not present in the experimental animals during the first 3 months of feeding, when the analyses were made. No evidence of infection was found. Forced feeding is admittedly an abnormal procedure, but since it led often to greater concentrations of urinary glucuronidase activity, it is possible that the amount of food ingested by a rat during the night preceding the collection period might have some influence on the amount of enzyme excreted during the following 24 hours.

With the same technique and the same person to express the urine, there was reasonable assurance that the bladder was emptied at the end of each collection period. The method of analysis yielded satisfactory reproducible results. The values were based on the amounts of glucuronidase activity recovered in 24 hours from the rat, rather than on per ml. of urine. Since the quantity of urine excreted is influenced by the amounts of fluid and food ingested, this comparison on the basis of the total 24-hour sample rather than on the volume of urine would seem to be the method of choice.

When food was withheld during the collection period, the average urinary β-glucuronidase activity excreted by young female rats of the Fischer strain maintained on the basal diet was approximately twice that of young females of the Buffalo strain fed the same diet ($P = 0.01$).

While the differences were not statistically significant, the Buffalo rats of the control groups fed 1 percent D- or L-tryptophan or 2 percent DL-tryptophan in the basal diet usually excreted more glucuronidase activity than the animals fed the unsupplemented basal diet. Forced feeding of the diets to rats of both strains usually resulted in a total urinary β-glucuronidase greater than that of the same rats when fasted. This effect of forced feeding was most striking with the DL-tryptophan-supplemented diet, but not with the diet containing indole. However, there was no evidence of an increase in urinary glucuronidase activity in the Buffalo- or the Fischer-strain rats, either with food withheld or with forced feeding, as a result of supplementing the diet containing fluorenylacetamide with 2 percent DL-tryptophan or with 0.8 percent indole; both of these supplements were associated with the appearance of bladder tumors in the Fischer-strain rats that survived on the diets for 9 to 11 months.

The range of variation is great in urinary β-glucuronidase activity of rats, but not any greater than that found by Boyland *et al.* (*1*). They reported that within groups of non-cancer patients, with infected urine, the range in activity for 24-hour samples of urine was 180 to 2,820 units among 16 patients, and for sterile urine 149 to 952 units among 25 patients. The amount of urinary β-glucuronidase activity excreted in 24 hours is obviously not proportional to the size of the body in different species. The extreme values were 15 to 5,496 units for rats maintained on the various diets, and averaged 928 and 761 for Buffalo and Fischer rats, respectively, with food withheld, and 2,054 and 1,941 units, with forced feeding, compared with values reported by Boyland *et al.* (*1*) for non-cancer patients, which varied from 180 to 2,820 units, average 586,[7] and for patients with multiple tumors of the bladder, which varied from 850 to 11,340 units, with an average of 3,504 units.[7] No values for urinary nitrogen were reported by Boyland *et al.*, but assuming an average hypothetical urinary nitrogen of 15 gm. in 24 hours, for man, the units of glucuronidase activity would vary from 0.012 to 0.19 per mg. of nitrogen for the non-cancer patients and 0.056 to 0.75 units per mg. urinary nitrogen for patients with cancer of the bladder. These values for man are low compared with the range of values, 0.1 to 50.0 units per mg. of urinary nitrogen, found among non-tumor-bearing rats.

The chromatographic studies show the effect of feeding supplementary DL-tryptophan and indole, in increased numbers and amounts of urinary metabolites (aromatic compounds). Boyland and Watson (*11*) reported the induction of bladder tumors in mice by implanting, in the bladder, paraffin or cholesterol pellets containing 3-hydroxyanthranilic acid. Allen *et al.* (*12*), using the same experimental procedure, induced bladder tumors in mice with cholesterol pellets of 3-hydroxykynurenine, 2-amino-3-hydroxyacetophenone, and 8-methoxykynurenic acid. These metabolites of tryptophan have been observed in human urine. 3-Hydroxyanthranilic

[7] Average values were not reported but were calculated by us.

acid was noted in increased amounts in the urine of patients who had cancer of the bladder (*13–15*).

Quagliariello *et al.* (*16*), in a preliminary report, noted a 50 percent decrease in the conversion *in vitro* of 3-hydroxyanthranilic acid (3-OH–AA) to quinolinic acid, by the liver of rats fed a diet containing 0.1 to 0.2 percent 2-FAA, for periods varying from 2 to 6 days, contrasted to livers of control rats that did not receive 2-FAA. The authors did not report the food intake of their animals, and it has been our experience that rats consume very small amounts of diets containing as little as 0.06 percent 2-FAA during the first few days after they are placed on such diets. Animals in a partial state of inanition are likely to have a much lower concentration of many enzymes (*17*) than animals that are fully fed. It seems hazardous, therefore, with the limited amount of data presented, for Quagliariello *et al.* to propose the presence of a biochemical lesion caused by 2-FAA so early in the cancer induction process. They postulated that inhibition of the conversion of 3-OH-AA to the *o*-quinoneiminic compound by 2-FAA may cause an accumulation of 3-OH-AA and of 3-hydroxykynurenine (3-OH-K), which under certain conditions leads to tumor formation. The accumulation of 3-OH-AA, 3-OH-K, or of 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid, however, was not demonstrated.

In this laboratory we (*9*) have observed an interference in the metabolism of a challenge dose of L-tryptophan in rats fed 0.06 percent *N*-2-fluorenylacetamide. Xanthurenic acid and several other metabolites of tryptophan accumulate in the urine, even when the diet of the animal contains 4 μg. of pyridoxine hydrochloride per gm., which is considered a generous amount, and which is sufficient to take care of the challenge dose of tryptophan when 2-FAA is not fed. The observed increase in urinary xanthurenic acid may be prevented by feeding the rat a large dose of pyridoxine hydrochloride during the experimental period when the tryptophan load is administered, thus suggesting that the fluorenylacetamide increases the requirement for vitamin B_6.

In the present study, 3-OH-AA was not detected in any of the urine. It was suspected of being present occasionally in the urine of rats fed several of the diets, including the basal diet F. After forced feeding of the DL-tryptophan-supplemented diet, the urine *infrequently* contained trace amounts of materials that might be identified by R_F, fluorescence, and/or staining as N^{α}-acetyl-3-hydroxykynurenine, 3-hydroxykynurenine-O-sulfate, 2-amino-3-hydroxyacetophenone, and 2-amino-3-hydroxyacetophenone-O-sulfate. Thus, while there was little evidence of the presence of *o*-hydroxyamino metabolites as a result of the use of nitrite in the Ekman test (*7*), traces of some of these compounds may have been present.

In view of the available experimental observations mentioned, it appears to be inadvisable to ascribe to the *o*-aminohydroxy metabolites of tryptophan an exclusive activity in the carcinogenic action of 2-FAA in the presence of supplementary tryptophan.

No evidence was obtained in these studies of rat urine to support

Boyland's (*1*, *15*) suggestion that urinary β-glucuronidase might release unconjugated *o*-aminohydroxy metabolites if the urine were retained in the bladder, because no hydrolysis of conjugates of metabolites of tryptophan or indole occurred in urine that was maintained at *p*H 5.9 to 6.2, and incubated for 20 hours at body temperature. As previously mentioned, aliquots of the urine incubated at a buffered *p*H 4.5 showed some hydrolysis of anthranilic acid glucuronide and of "compound B," but when bacterial β-glucuronidase was added to the urine buffered before incubation at *p*H 6, there was almost complete hydrolysis of these conjugates, as well as the disappearance of other unidentified materials that were considered to be glucuronides.

It is of course possible that one or more of the trace metabolites of DL-tryptophan exerts a deleterious effect on the bladder epithelium and thus acts as a cocarcinogen with 2-FFA. Another possibility is that the induction of bladder cancer by *N*-2-fluorenylacetamide, when DL-tryptophan or indole is incorporated in the diet, may be due to the continued exposure of the bladder epithelium to the increased number and amounts of aromatic metabolites of indole and tryptophan, which are excreted by the kidneys during the time that metabolites of *N*-2-fluorenylacetamide are also present.

The amounts of metabolites observed in the chromatograms, even of those compounds that appeared in relatively large amounts, were actually very small and could account for a low percentage of the 64 mg. of DL-tryptophan or the 25 mg. of indole present in the amount of supplemented force-fed food. Nevertheless the total increase in urinary aromatic metabolites resulting from feeding the supplements may be sufficient to exert some deleterious influence on the bladder epithelium. Brown and Price (*18*) studied this problem by using a challenging dose of L-tryptophan. They found a low excretion of tryptophan metabolites in the cat, a species reported to have a low incidence of bladder cancer (*19*), and a relatively high level of urinary tryptophan metabolites in man, the dog, and the rat, species that are susceptible to bladder cancer. They suggested that these correlations, together with the apparent relation of tryptophan metabolism to the induction of bladder cancer in rats, with fluorenylacetamide (*3*, *20*), and the relatively large excretion of kynurenine, kynurenic acid, and acetylkynurenine by many patients with bladder cancer, after ingestion of tryptophan (*21*), present an interesting area for speculation concerning the possible etiology of this type of neoplasm. For a discussion of many aspects of the experimental induction of tumors of the bladder *see* Bonser, Clayton, and Jull (*22*), and Boyland (*15*).

REFERENCES

(*1*) BOYLAND, E., WALLACE, D. M., and WILLIAMS, D. C.: The activity of the enzymes sulphatase and β-glucuronidase in the urine, serum and bladder tissue. Brit. J. Cancer 9: 62–79, 1955.

(2) DUNNING, W. F., CURTIS, M. R., and MAUN, M. E.: The effect of added dietary tryptophane on the occurrence of 2-acetylaminofluorene-induced liver and bladder cancer in rats. Cancer Res. 10: 454–459, 1950.

(3) DUNNING, W. F., and CURTIS, M. R.: The role of indole in the incidence of 2-acetylaminofluorene-induced bladder cancer in rats. Proc. Soc. Exper. Biol. & Med. 99: 91–95, 1958.

(4) TALALAY, P., FISHMAN, W. H., and HUGGINS, C.: Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. J. Biol. Chem. 166: 757–772, 1946.

(5) DALGLIESH, C. E.: Isolation and examination of urinary metabolites containing an aromatic system. J. Clin. Path. 8: 73–78, 1955.

(6) ———: Two-dimensional paper chromatography of urinary indoles and related substances. Biochem. J. 64: 481–485, 1956.

(7) EKMAN, B.: Paper chromatography of primary aromatic amines. Acta chem. scandinav 2: 383–384, 1948.

(8) MANTEL, N.: Rapid estimation of standard errors of means for small samples. Am. Statistician 5: 26–27, 1951.

(9) DYER, H. M., and MORRIS, H. P.: An effect of *N*-2-fluorenylacetamide on the metabolism of L-tryptophan in rats. (Abstracts.) Am. Chem. Soc., 131st Meet., Miami, Florida, No. 21, 10c, 1957.

(10) LEVVY, G. A.: Glucuronide metabolism, with special reference to the steroid hormones. Vitamins & Hormones 14: 283, 1956.

(11) BOYLAND, E., and WATSON, G.: 3-Hydroxyanthranilic acid, a carcinogen produced by endogenous metabolism. Nature, London 177: 837–838, 1956.

(12) ALLEN, M. J., BOYLAND, E., DUKES, C. E., HORNING, E. S., and WATSON, J. G.: Cancer of the urinary bladder induced in mice with metabolites of aromatic amines and tryptophan. Brit. J. Cancer 11: 212–228, 1957.

(13) BOYLAND, E., WALLACE, D. M., and WILLIAMS, D. C.: The activity of the enzymes sulphatase and glucuronidase in the urine of normal and cancer patients. Biochem. J. 56: 29, 1954.

(14) ———: The metabolism of tryptophan; metabolism of tryptophan in patients suffering from cancer of the bladder. Biochem. J. 64: 578–582, 1956.

(15) BOYLAND, E.: The biochemistry of cancer of the bladder. Brit. M. Bull. 14: 153–158, 1958.

(16) QUAGLIARIELLO, E., AURICCHIO, S., and RINALDI, E.: Conversion of 3-hydroxyanthranilic acid to quinolinic acid in presence of rat liver treated with 2-acetylaminofluorene. Nature, London 181: 624, 1958.

(17) VAN PILSUM, J. F., SPEYER, J. F., and SAMUELS, L. T.: Essential amino acid deficiency and enzyme activity. Arch. Biochem. 68: 42–53, 1957.

(18) BROWN, R. R., and PRICE, J. M.: Quantitative studies on metabolites of tryptophan in the urine of the dog, cat, rat, and man. J. Biol. Chem. 219: 985–997, 1956.

(19) BLOOM, F.: Pathology of the Dog and Cat: The Genitourinary System, with Clinical Considerations. Evanston, Am. Vet. Publ., Inc., 184, 1954, 463 pp.

(20) BOYLAND, E., HARRIS, J., and HORNING, E. S.: The induction of carcinoma of the bladder in rats with acetamidofluorene. Brit. J. Cancer 8: 647–654, 1954.

(21) BROWN, R. R., PRICE, J. M., and WEAR, J. B.: The metabolism of tryptophan in bladder tumor patients. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 7, 1955.

(22) BONSER, G. M., Clayton, D. B., and JULL, J. W.: Some aspects of the experimental induction of tumours of the bladder. Brit. M. Bull. 14: 146–152, 1958.

Walker Carcinosarcoma 256 Tissue as a Dietary Constituent. III. Sodium as a Factor in Stimulation of Water and Food Intake and Growth in Tumor-Bearing Rats [1]

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SUMMARY

Lyophilized tumor tissue used as a source of nitrogen in the diet can stimulate food intake and weight gain in tumor-bearing rats that have reached a weight plateau on a 20 percent casein diet. Water-soluble and water-insoluble fractions of lyophilized, defatted Walker carcinosarcoma 256 tissue were prepared, incorporated into diets, and fed to rats bearing Walker 256 tumors. The stimulatory properties of these fractions were compared to those of whole tumor tissue. The water-insoluble fraction produced a delayed stimulation resulting in less eventual body-weight gain but equal tumor weights. A marked stimulation by the water-soluble fraction was observed similar to that of whole tumor. This activity was due to the Na ion content. Increasing the Na content of the C-20 diet by added NaCl resulted in markedly increased water intake, food intake, and weight gain, less adrenal hypertrophy, larger tumors, and increased hydration of the carcass. Whole tumor tissue showed these same effects on a somewhat lower food intake. On a pair-fed basis, whole tumor tissue showed a greater growth stimulation than C-20 + NaCl, which suggests that there may be an additional factor in tumor tissue besides the Na ion.—J. Nat. Cancer Inst. 24: 197–209, 1960.

SPRAGUE-DAWLEY rats bearing the Walker carcinosarcoma 256 and ingesting a 20 percent casein diet ultimately exhibit a marked decrease in food intake, and fail to gain weight. Stimulation of food intake and growth (weight gain) in such animals can be demonstrated by replacing the casein in the diet with lyophilized Walker 256 tumor tissue (*1*). Lyophilized tissue from other tumors (rat, mouse, and human), when substituted for casein, showed this effect, though the degree of stimulation varied (*2*). Extraction of tumor tissue with fat solvents resulted in a defatted product that showed no loss in activity (*3*). Haven and Bloor have reported stimulation of growth and food intake in tumor-bearing rats fed a phospholipide fraction of Walker 256 tumor tissue (*4*).

This communication presents data on the stimulation of food and

[1] Received for publication July 21, 1959.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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water intake and growth by fractions prepared from defatted Walker 256 tumor tissue.

MATERIALS AND METHODS

Preparation of fractions.—Procedures for the lyophilization and defatting of Walker 256 tumor tissue have been previously described (*1*, *2*). The lyophilized, defatted tissue was extracted 5 times with boiling distilled water for periods of 20 minutes each, with the use of 3.75, 1.5, 1.5, 1.5, and 1.0 liters per kg. for successive extractions. The residue was washed with alcohol and ether consecutively and air-dried. The water-insoluble product (H_2O-I) contained 13 percent nitrogen and 3 percent ash and represented about 80 percent of the defatted tissue. The water extracts were combined and lyophilized. Considerable foaming occurred, but this could be minimized by keeping the flasks in a dry-ice bath. The water-soluble product, referred to as H_2O-S, was a nonhygroscopic, light-yellow solid that could be easily powdered. It contained 11 percent nitrogen and 22 percent ash.

Experimental conditions.—Tumor fractions or known compounds were incorporated into diets or fed as supplements to the 20 percent casein diet, C-20. Both C-20 and a diet (T-24) containing 24 percent lyophilized Walker 256 tumor tissue were used as standards for comparison. The composition of the C-20 and T-24 diets has been given previously (*1*). All diets were essentially isocaloric and isonitrogenous. Adjustments for differences in nitrogen were made by varying the quantity of starch in the diets.

The experimental diets were fed to rats bearing the transplanted Walker 256 tumor. In previous experiments (*1*, *2*) animals had been placed on the diets when the tumor became palpable (about 7 days) or even earlier. In this report, feeding experiments were started 15 days after tumor transplantation. At this time those rats were selected whose tumors appeared relatively uniform in size and weight and whose body weights were essentially the same. Of 100 inoculated rats, 50 could thus be initially selected. Animals that later developed ulcerated tumors or respiratory infections during the course of the experiments were discarded.

Two types of feeding procedures were used. In one, all animals were fed the C-20 diet until they failed to gain weight. The time at which this weight plateau was reached varied considerably among individuals. When each rat reached the plateau, it was given an experimental diet or supplement and the effect on changes in weight and food intake was observed. In the second procedure, comparisons were made with groups of animals ingesting the experimental and control diets throughout the course of the experiment. Each diet group contained 15 to 20 rats at the start of the experiment; 6 to 10 remained in satisfactory condition at the end.

Food intake and weight change were recorded daily. At the end of all experiments, animals were killed by cervical dislocation, and tumors were

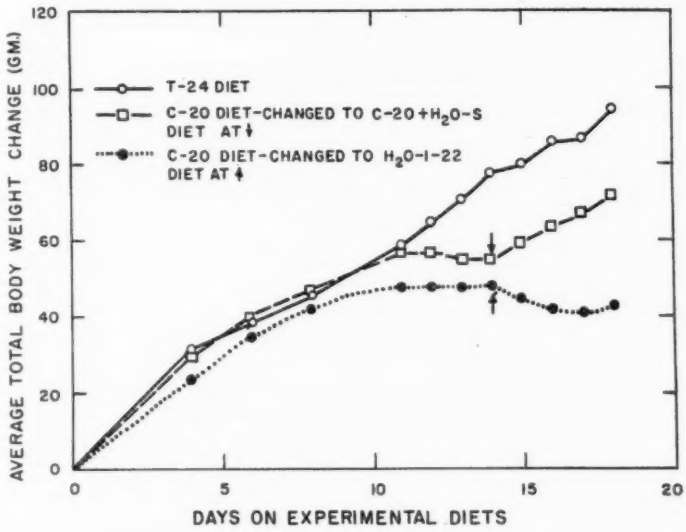
surgically removed and weighed. In one experiment, carcasses (minus adrenals, livers, and intestinal contents) and tumors were weighed and lyophilized. Adrenals were weighed separately. Aliquots of carcass and tumor were defatted with 3:1 alcohol-ether followed by 1:1 chloroform-methanol. The residues were washed with alcohol-ether and lyophilized. The weight of the lyophilized residue represented the fat-free dry weight. Fat content was calculated by difference.

RESULTS AND DISCUSSION

Fractions of Tumor Tissue

In testing the two fractions H_2O-I and H_2O-S for activity, we incorporated them as components in the diet; H_2O-I was used as a nitrogen source at the 22 percent level (H_2O-I-22). H_2O-S was added to the casein control diet C-20, at a concentration (4.5%) comparable to its level in whole tumor-tissue diet.

These two fractions were compared in tests with rats that had reached a plateau on C-20. A typical experiment with 3 rats per diet is shown in text-figure 1. When the water-insoluble diet (H_2O-I-22) replaced C-20 on the 14th day, there was no ensuing increase in growth or food intake. However, addition of H_2O-S to C-20 produced an increase in weight parallel to that of animals that had been fed the whole tumor diet (T-24) throughout the experiment. This indicated that a growth-stimulating factor



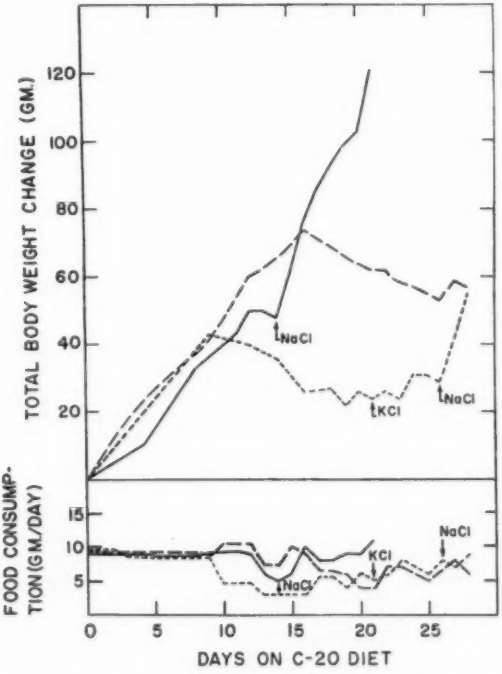
TEXT-FIGURE 1.—Effect of ingested H_2O-S and H_2O-I fractions of Walker tumor tissue on total body-weight change of tumor-bearing rats that have reached a weight plateau on the C-20 diet.

resided in the water-soluble fraction. On the other hand, under the conditions chosen, the H_2O-I fraction was ineffective.

Since the H_2O-S fraction of tumor tissue had shown a definite growth stimulation of rats that had attained a weight plateau on C-20, we centered our attention on this fraction. When an alcohol extract of H_2O-S was put through a charcoal column, the forerun yielded a product that contained only 3 percent nitrogen and had a high inorganic salt content (13% Na and 13% K). This inorganic fraction, fed as a supplement, produced a weight change of 27 gm. overnight in a rat that had previously reached a plateau on the C-20 diet. Most of the weight change was due to an increased water intake as a result of ingestion of the salt fraction.

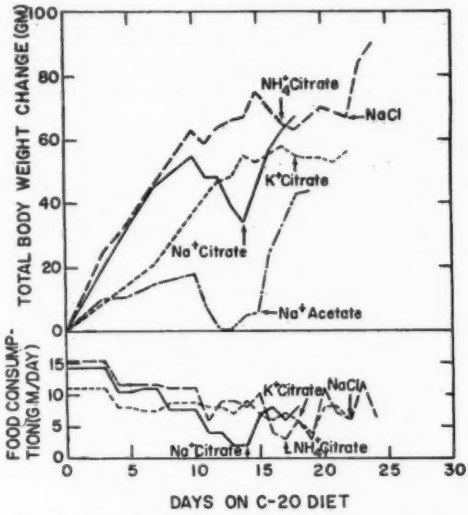
Inorganic Ion Supplementation

A variety of inorganic salts, fed as supplements at the level of 0.1 to 0.2 gm. per day, was tested with the use of animals that had attained a plateau on the C-20 diet. Some of these tests are shown in text-figures 2 and 3. It can be seen (text-fig. 2) that there was an immediate and marked stimulation in growth as a result of increased food and water intake when the supplement was sodium chloride. Potassium chloride,



TEXT-FIGURE 2.—Effect of NaCl and KCl supplements upon total body-weight change and food consumption of Walker 256 tumor-bearing rats that have reached a weight plateau on the C-20 diet.

on the other hand, had no effect. Furthermore, if one replaced the KCl by NaCl, a marked stimulation in growth (largely due to increased water intake) occurred. Calcium acetate, calcium citrate, magnesium citrate and magnesium carbonate (not shown), and ammonium citrate (text-fig. 3) were ineffective. This suggested that possibly only NaCl was capable of producing this effect. Sodium citrate and sodium acetate (text-fig. 3) were as effective as NaCl.



TEXT-FIGURE 3.—Effect of Na^+, K^+, and NH_4^+ salts upon total body-weight change and food consumption of Walker 256 tumor-bearing rats that have reached a weight plateau on the C-20 diet.

These data suggested that the Na^+ ion was the specific cation responsible for the stimulating effect. Coincidental with the increased intake of Na^+, an increased intake of H_2O and increased output of urine were observed. Administration of combinations of ions, such as Na^+ in conjunction with Ca^{++} or Mg^{++}, has not been tried.

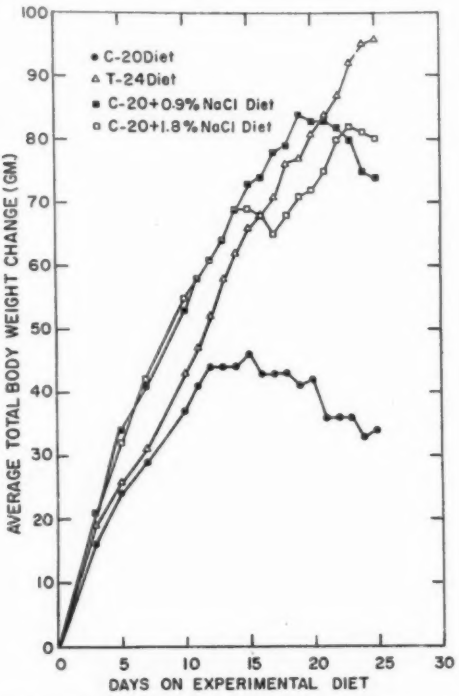
Sodium Chloride Additions to Diet

Effect on growth.—All our experimental and control diets include a salt mixture (*5*) adequate for optimal growth of normal rat. This salt mixture supplies 0.3 percent NaCl. The effect of additional dietary sodium chloride on total body weight of tumor-bearing rats, and on weight change and composition of carcass and tumor, was studied at 2 levels of added sodium chloride, 0.9 and 1.8 percent of the diet. The 0.9 percent level represented the amount of NaCl added to the C-20 diet to make it equivalent to the T-24 diet in Na content. (Lyophilized Walker 256 tumor contained 1.5% Na.) The salt was incorporated into the C-20 diet at

the expense of an equal amount of starch. The diets are referred to as C-20 + 0.9 percent NaCl and C-20 + 1.8 percent NaCl. Diets C-20 and T-24 were used as controls.

These 4 diets were fed *ad libitum* to animals bearing 15-day-old tumors as shown in text-figure 4. The 2 salt-containing diets were as effective as T-24 for the greater part of the experiment—19 to 23 days. Increasing the salt content twofold had no increased effect. Food intake and total body, tumor, and adrenal weights on the 4 regimens are shown in table 1. Similar data from normal rats and from a control group of rats bearing 15-day tumors are also shown. The total body weight (including tumor) and the weight of the tumor were greatest for the group on the T-24 diet, although food intake was greatest for animals receiving salt diets.

In a separate experiment, food intake of tumor-bearing rats on the C-20 + 1.8 percent NaCl diet was restricted by pair-feeding them to a group ingesting T-24. Differences in growth rates of the two groups are shown in text-figure 5. Thus it was the increased food intake by animals ingesting the salt diets *ad libitum* that produced growth rates matching that of T-24 (text-fig. 4). Although NaCl can effectively prevent the loss of appetite and weight gain to a large extent, apparently it cannot duplicate tumor-tissue effects completely.



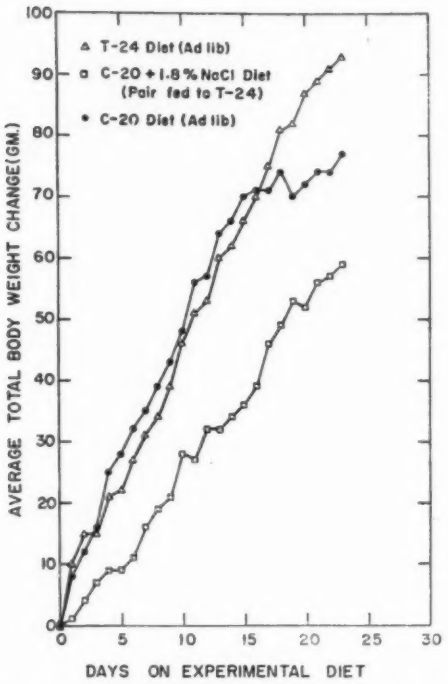
TEXT-FIGURE 4.—Effect of additions of NaCl to the C-20 diet upon total body-weight change of Walker 256 tumor-bearing rats.

TABLE 1.—Total body weight, tumor weight, adrenal weight, and food consumption of Walker 256 tumor-bearing rats ingesting the C-20, T-24, C-20 + 0.9 percent NaCl, and C-20 + 1.8 percent NaCl diets *ad libitum* (mean ± standard error*)

Group	Number of rats	Food intake (*gm./day*)	Initial total body weight (*gm.*)	Final total body weight (*gm.*)	Tumor weight (*gm.*)	Adrenal weight (*mg./100 gm. total body weight*)
Normal control	4	—	—	345 ± 12	—	14 ± 1
Normal control	9	—	—	231 ± 6	—	13 ± 1
Initial tumor-bearing control†	6	—	257 ± 4	—	8 ± 1	16 ± 1
C-20 diet	8	8.2 ± 0.4	258 ± 6	292 ± 8	116 ± 3	45 ± 4
T-24 diet	9	8.5 ± 0.5	255 ± 4	350 ± 13	153 ± 14	29 ± 2
C-20 + 0.9 percent NaCl diet	6	10.4 ± 0.6	260 ± 7	335 ± 16	130 ± 12	27 ± 2
C-20 + 1.8 percent NaCl diet	10	9.7 ± 0.4	256 ± 5	337 ± 16	136 ± 10	26 ± 1

*Standard error = $\frac{\text{range of values}}{\text{number of animals}}$ (*6*).

†Rats bearing 15-day tumors and killed at start of experiment.



TEXT-FIGURE 5.—Total body-weight change of Walker 256 tumor-bearing rats ingesting the C-20 and T-24 diets (*ad libitum*) and the C-20 + 1.8 percent NaCl diet (pair-fed to the T-24 group).

Adrenal weights (mg./100 gm. total body weight) of the various groups of control and experimental animals are shown in table 1. The initial control group bearing 15-day-old tumors suggests a slight increase in adrenal weight. However, there was a striking increase in the 4 diet groups bearing large tumors. Adrenal hypertrophy has been reported previously as a feature of tumor growth (*7–9*). Begg has thought this hypertrophy to be related to adrenal hyperfunction (*10*). Our data indicate that the degree of adrenal hypertrophy was related to the NaCl content of the diet. There was considerably less hypertrophy in the 2 groups receiving added NaCl and the group on T-24. It is probable that the Na of dietary tumor tissue was responsible for this effect in the group of rats ingesting T-24.

We have made a histological examination of adrenals from tumor-bearing rats that had ingested either the C-20 diet or C-20 supplemented with salt. A qualitative comparison showed that the *zona glomerulosa* in the C-20 group was appreciably wider and had a higher content of lipide, as judged from Oil Red O stain.[3] Deane *et al.* have described enlargement of the *zona glomerulosa* in normal rats as a result of feeding a sodium-

[3] We are indebted to Dr. Frederic C. Bartter of the National Heart Institute for the interpretation of these sections.

deficient diet (*11*). Our results suggest that the adrenal hypertrophy in tumor-bearing rats is related to an increased demand for salt.

Effect on carcass and tumor composition.—The final composition of tumor and carcass of rats ingesting the C-20, T-24, and C-20 + 1.8 percent NaCl diets *ad libitum* is given in table 2. Coincident with tumor increases in all 3 groups were large losses in carcass weight which could be due to water, fat-free dry component, fat, or combinations of these.

When the figures for water as a percentage of fat-free wet weight (table 2) were compared, the water content of carcasses on the T-24 and C-20 + 1.8 percent NaCl was higher than on C-20. Tumors contained more water than carcasses, but there was no apparent influence of diet on the water content of tumor.

The difference in hydration of carcasses was also discernible at autopsy. Tumor-bearing animals on the C-20 diet appeared dehydrated, and tissues were especially dry in the area of the tumor. The tissues of animals on the T-24 and C-20 + 1.8 percent NaCl diets were well hydrated.

Occasionally a salt-fed animal showed a curious deposition of extracellular water in the loose connective tissue. This water was sometimes distributed generally and sometimes located in the mesentery or axillary region. It should be emphasized that animals receiving additional salt showed a marked increase in water consumption. The evidence suggests a disturbance in the water and electrolyte metabolism of these tumor-bearing rats. Electrolyte-balance studies will be reported in another communication.

Evidence for Possible Factor in Water-Insoluble Fraction

Short-term tests of the water-insoluble fraction (H_2O-I) showed no evidence of growth stimulation in this fraction (text-fig. 1). A preliminary study, however, had indicated that H_2O-I, when incorporated into the diet as a source of nitrogen, supported the growth of young, non-tumor-bearing animals as well as the C-20 or T-24 diets. Therefore, a more extensive test of the H_2O-I fraction in tumor-bearing animals was undertaken. Three groups of rats bearing 15-day tumors were placed on C-20, T-24, and H_2O-I-22, respectively, and maintained on these diets for 25 days (text-fig. 6). The animals on C-20 reached a plateau, those on T-24 contined to grow, and the animals on H_2O-I-22 showed a delay in growth response and then assumed the growth rate of animals on the T-24 diet (table 3). This period of delay was associated with a decrease in food intake (1–2 gm./day less than the average of 7–8 gm./day for the total period). The above experiment was repeated 3 times with essentially the same results.

A comparison of the tumor weights showed that rats on H_2O-I-22 grew tumors equal to or larger than those of animals on T-24. Both groups had considerably larger tumors than those of the C-20 animals. However, the carcass-weight loss was greatest in the animals on the H_2O-I diet. The total weight gain was distributed differently in spite

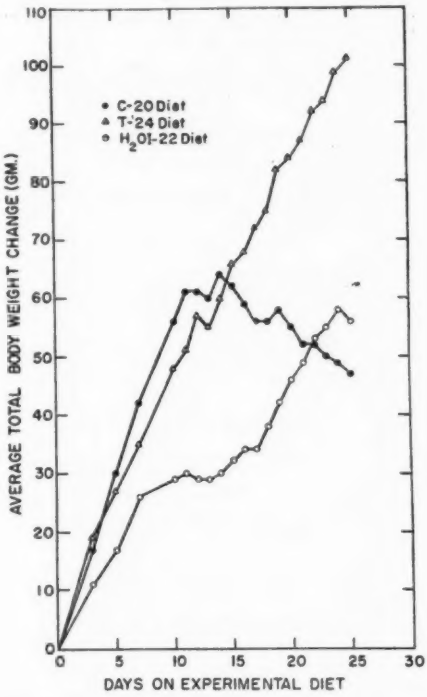
TABLE 2.—Carcass and tumor composition of Walker 256 tumor-bearing rats ingesting the C-20, T-24, and C-20 + 1.8 percent NaCl diets *ad libitum* (mean ± standard error*)

		Carcass					Tumor				
Group	Number of rats analyzed	Wet weight† (*gm.*)	H_2O (*gm.*)	Fat-free dry weight (*gm.*)	Fat (*gm.*)	$\frac{H_2O}{\text{Fat-free wet weight}}$ (*percent*)	Wet weight (*gm.*)	H_2O (*gm.*)	Fat-free dry weight (*gm.*)	Fat (*gm.*)	$\frac{H_2O}{\text{Fat-free wet weight}}$ (*percent*)
Initial control											
C-20 diet	6	226 ± 3	152 ± 2	52.2 ± 1.6	22.3 ± 1.3	74.4 ± 0.3	8 ± 1	7‡	0.9‡	0.2‡	88.0‡
T-24 diet	8	155 ± 5	109 ± 4	40.4 ± 1.3	5.3 ± 0.4	73.0 ± 0.4	116 ± 3	98 ± 3	13.9 ± 0.6	3.6 ± 0.1	87.8 ± 0.2
C-20 + 1.8 percent NaCl diet	8	169 ± 10	124 ± 7	39.2 ± 3.0	6.0 ± 1.2	76.2 ± 0.6	143 ± 10	123 ± 8	15.8 ± 0.8	4.4 ± 0.4	88.5 ± 0.3
	9	172 ± 9	123 ± 6	41.8 ± 2.3	6.7 ± 1.6	74.7 ± 0.5	128 ± 5	109 ± 5	15.1 ± 0.4	4.1 ± 0.2	87.7 ± 0.4

*Standard errors calculated as in table 1.
†After removal of adrenals, liver, and intestinal contents.
‡Combined for analysis.

of the fact that total food intake of the 3 groups was essentially the same and all 3 diets were approximately isocaloric, isonitrogenous, and equally effective in supporting growth of normal animals. These results suggested that the H_2O-I fraction under conditions of prolonged feeding also contributed to stimulation of food intake and tumor growth.

The incorporation of 0.9 percent NaCl into the H_2O-I-22 diet (experiment D, table 3) resulted in total body-weight changes and tumor weights somewhat greater than those produced by T-24.



TEXT-FIGURE 6.—Total body-weight change of Walker 256 tumor-bearing rats ingesting the C-20, T-24, and H_2O-I-22 diets (experiment A).

The data indicate that the dietary factor in tumor tissue principally responsible for the maintenance of water intake, food intake, and weight gain in tumor-bearing rats is the sodium ion. It also can restore water and food intake and weight gain to animals that have reached a plateau on the C-20 diet. Some of the evidence presented indicates that there may be an additional stimulatory component in tumor tissue: (*a*) The effect of the H_2O-I fraction on tumor growth could not be due to sodium ion, since H_2O-I had a low ash content; (*b*) casein diets with added sodium did not completely duplicate the effect of T-24 for extended periods.

TABLE 3.—Weight changes and food consumption of Walker 256 tumor-bearing rats ingesting the C-20, T-24, and H_2O-I-22 diets (mean ± standard error*)

Experiment	Number of rats	Days on diet	Diet	Total experimental period: Food intake (gm./day)	Total body weight: Initial (gm.)	Total body weight: Final (gm.)	Total body weight: Change (gm.)	Tumor weight (gm.)	Carcass-weight change (gm.)	Final part of experimental period †: Food intake (gm./day)	Final part of experimental period †: Total body-weight change (gm./day)
A	9	25	C-20	8.3 ± 0.3	259 ± 4	306 ± 8	47 ± 5	124 ± 3	−77 ± 5	5.6 ± 0.4	−1.6 ± 0.6
	10		T-24	9.0 ± 0.4	254 ± 6	354 ± 9	100 ± 6	140 ± 7	−40 ± 8	8.2 ± 0.4	3.4 ± 0.6
	9		H_2O-I-22	8.0 ± 0.4	256 ± 7	313 ± 10	57 ± 7	139 ± 5	−82 ± 5	7.7 ± 0.4	2.4 ± 0.3
B	9	25	C-20	8.1 ± 0.4	246 ± 7	291 ± 14	45 ± 9	106 ± 5	−61 ± 11	5.9 ± 0.6	−0.5 ± 0.5
	6		T-24	7.8 ± 0.4	246 ± 4	319 ± 10	73 ± 11	126 ± 10	−53 ± 12	6.7 ± 0.4	2.2 ± 0.9
	10		H_2O-I-22	7.6 ± 0.4	240 ± 6	297 ± 8	57 ± 6	127 ± 5	−70 ± 8	7.2 ± 0.2	2.5 ± 0.3
C	7	25	C-20	7.8 ± 0.5	235 ± 8	278 ± 12	43 ± 8	112 ± 4	−69 ± 8	5.8 ± 0.3	−0.1 ± 0.4
	8		T-24‡	7.7 ± 0.3	245 ± 6	316 ± 8	71 ± 5	138 ± 8	−67 ± 9	7.6 ± 0.3	3.4 ± 0.8
	9		H_2O-I-22‡	7.7 ± 0.1	245 ± 5	299 ± 6	54 ± 6	144 ± 9	−90 ± 7	7.6 ± 0.5	2.7 ± 0.7
D	9	20	C-20	8.7 ± 0.5	254 ± 10	279 ± 9	25 ± 10	93 ± 7	−68 ± 15	5.8 ± 0.9	−2.6 ± 1.6
	9		T-24	8.4 ± 0.6	266 ± 3	330 ± 12	64 ± 14	119 ± 8	−55 ± 10	7.6 ± 0.4	2.9 ± 0.8
	8		H_2O-I-22	7.5 ± 0.5	252 ± 7	297 ± 12	45 ± 14	133 ± 7	−88 ± 7	7.6 ± 0.4	3.0 ± 0.6
	9		H_2O-I-22+.09 percent NaCl	8.4 ± 0.4	268 ± 8	364 ± 19	96 ± 16	162 ± 13	−66 ± 7	9.1 ± 0.4	5.1 ± 1.3

*Standard errors calculated as in table 1.

†Last 10 days of experiments A, B, and C; last 5 days of experiment D.

‡First 5 days on C-20.

REFERENCES

(1) MILLAR, F. K., WHITE, J., BROOKS, R. H., and MIDER, G. B.: Walker carcinosarcoma 256 tissue as a dietary constituent. I. Stimulation of appetite and growth in the tumor-bearing rat. J. Nat. Cancer Inst. 19: 957–967, 1957.

(2) MILLAR, F. K., BROOKS, R. H., and WHITE, J.: Stimulation of appetite and growth in the tumor-bearing rat by a variety of tissues. J. Nat. Cancer Inst. 22: 685–696, 1959.

(3) MILLAR, F. K., and WHITE, J.: Tumor tissue as a dietary constituent for the tumor-bearing rat. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 327, 1958.

(4) HAVEN, F. L., and BLOOR, W. R.: Tumor-host relationships: A lipid appetite factor. Fed. Proc. 14: 223–224, 1955.

(5) HUBBELL, R. B., MENDEL, L. B., and WAKEMAN, A. J.: A new salt mixture for use in experimental diets. J. Nutrition 14: 273–285, 1937.

(6) MANTEL, N.: Rapid estimation of standard errors of means for small samples. Am. Statistician 5: 26–27, 1951.

(7) SAVARD, K.: Adrenal changes in animals bearing transplanted tumors. Science 108: 381–382, 1948.

(8) HAVEN, F. L., BLOOR, W. R., and RANDALL, C.: Lipids of the carcass, blood plasma, and adrenals of the rat in cancer. Cancer Res. 9: 511–514, 1949.

(9) BEGG, R. W.: Systemic effects of tumors in rats. Cancer Res. 11: 341–344, 1951.

(10) ———: Tumor-host relations. Advances Cancer Res. 5: 1–54, 1958.

(11) DEANE, H. W., SHAW, J. H., and GREEP, R. O.: The effect of altered sodium or potassium intake on the width and cytochemistry of the zona glomerulosa of the rat's adrenal cortex. Endocrinology 43: 133–153, 1948.

Quantitative Nutritional Studies With Water-Soluble, Chemically Defined Diets. X. Formulation of a Nutritionally Complete Liquid Diet [1]

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SUMMARY

The formulation of a nutritionally adequate, chemically defined, crystal-clear liquid diet composed of 18 crystalline L-amino acids (except for glycine), the water-soluble vitamins, the pertinent salts, glucose, the fat-soluble vitamins, and ethyl linoleate as the source of essential fatty acid, has been achieved. This liquid diet is prepared as a transparent, clear, stable emulsion of the latter two, fat-soluble, ingredients in a 50 percent solution of the other ingredients in water, with the aid of a small amount of polyoxyethylene (20) sorbitan monoöleate (Tween 80). The lipide phase reveals no tendency to separate or become rancid, and the clear diet may be stored at room temperature or at −20° C. for prolonged periods without apparent deterioration. Weanling rats fed these diets *ad libitum* for over 3 months exhibited no signs of essential fatty-acid deficiency and, indeed, grew at the same rate as did controls that were provided the fat-soluble vitamins in Mazola (corn) oil as a supplement.—J. Nat. Cancer Inst. 24: 211–219, 1960.

PREVIOUS STUDIES in this laboratory (*1–9*) were concerned with the development of water-soluble, chemically defined diets. There were two primary aims: first, to ascertain the effect of variations in such diets on experimental animals under conditions of relatively high quantitative precision and, second, to formulate diets which would have potential application both to the alimentation of patients to whom large quantities of calorigenic materials had to be administered *via* parenteral means and to the study of patients suffering from such pathologic conditions as ulcerative colitis, kwashiorkor, and certain metabolic dysfunctions, *e.g.*, phenylpyruvic oligophrenia. Toward this end, liquid diets were developed which permitted the institution of long-term quantitative nutritional studies with normal and tumor-bearing animals. The diets, which were composed of crystalline essential and nonessential amino acids, the water-soluble vitamins, the required salts and glucose, were provided as 50 per-

[1] Received for publication July 31, 1959.
[2] Deceased, February 12, 1959.
[3] The authors are grateful to Mrs. Elizabeth M. Thomas and Mrs. Nellie L. Washington for their technical assistance.
[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

cent solutions in water; a source of essential fatty acid, in which the fat-soluble vitamins were dissolved, was supplied as a separate supplement. Cognizance was taken early of the fact that such diets had a serious defect in that the fats and fat-soluble vitamins could be neither adequately homogenized nor mixed uniformly with the concentrated aqueous phase, and hence had to be provided independently (*1*). Another defect accrued from the initial use in the supplement of ethyl linoleate which, though it could serve in a dual capacity as a source of essential fatty acid and as a solvent for the fat-soluble vitamins, had the distinct disadvantage of marked susceptibility to rapid autoxidation and required daily renewal. The ethyl linoleate component was therefore replaced with the more stable Mazola (corn) oil, which, however, introduced a further undesirable feature in that it was now the only ingredient in the diet chemically undefined.

With these facts in mind, studies were undertaken with the ultimate goal of devising a nutritionally adequate liquid diet which would incorporate all the essential fatty-acid and fat-soluble components and which would, at the same time, be composed solely of ingredients whose chemical constitution had been unequivocally established. The formulation of such a diet is the primary concern of the present communication.

MATERIALS AND METHODS

Soluble Diets

The various diets were prepared as 50 percent solutions in water from dietary components of the same quality as those in the first study of this series (*1*). In every instance, the glucose, vitamin, salt, and fat-soluble component composition, per kg. of dry diet or per 2 liters of a 50 percent aqueous solution thereof, was identical with that given in table 1. Each of the diets contained, in addition, the 10 essential L-amino acids, 8 non-essential amino acids (all of the L-configuration with the exception of glycine), ethyl linoleate,[5] and Polysorbate 80[6] in the amounts given.

Diet #26.—This diet is prepared as described previously (*1*) and, with the exception of the fats and fat-soluble vitamins which are provided as a separate supplement, has essentially the same composition per kg. of dry diet as that given for diet #86.

Diet #86.—One kg. of the dry diet contains all the components listed in table 1 in addition to the 10 essential amino acids (7.5 gm. L-arginine·HCl, 5.4 gm. L-histidine·HCl·H_2O, 5.0 gm. L-isoleucine, 8.0 gm. L-leucine, 12.4 gm. L-lysine·HCl, 6.0 gm. L-methionine, 9.0 gm. L-phenylalanine, 5.0 gm. L-threonine, 2.0 gm. L-tryptophan, and 7.0 gm. L-valine, 8 non-essential amino acids or derivatives thereof [7.5 gm. L-alanine, 15.9 gm. L-aspartic acid (added as an aqueous solution of the monosodium salt),

[5] A product of the Hormel Research Foundation, Austin, Minn.

[6] A U.S.P. grade of Tween 80 [polyoxyethylene (20) sorbitan monoöleate], which was kindly provided by Mr. Rolfe Silverman of the Atlas Powder Co., Wilmington, Del.

TABLE 1.—Basal composition of diets*†

Water-soluble components				Fat-soluble components	
Vitamins‡	Weight (*mg.*)	Salts	Weight (*gm.*)		
Thiamine·HCl	5. 0	Glucono-δ-lactone§	26. 09	Vitamin A acetate‖	10.0 mg·
Riboflavine	7. 5	Magnesium oxide§	0. 73	Calciferol‖	7.0 μg·
Pyridoxine·HCl	6. 3	Potassium hydroxide§	6. 17	α-Tocopherol acetate‖	50.0 mg·
Niacin	37. 5	Ferrous gluconate§	1. 72	2-Methyl-1 : 4-napthoquinone‖	4.2 mg·
Inositol	250. 0	Manganous acetate·$4H_2O$¶	0. 26		
Choline·HCl	2500. 0	Cupric acetate·H_2O¶	0. 015		
Calcium pantothenate	50. 0	Cobaltous acetate·$4H_2O$¶	0. 009		
Biotin	0. 3	Zinc benzoate¶	0. 022		
Folic acid	0. 5	Ammonium molybdate·$4H_2O$¶	0. 006		
Ascorbic acid	500. 0	Potassium iodide¶	0. 03		
Vitamin B_{12}	0. 1	Monocalcium fructose 1,6-diphosphate	50. 5		
p-Aminobenzoic acid	300. 0				

*Quantities listed refer to the preparation of 1 kg. of dry diet or 2 liters of a 50% solution.

†The total weight, after the addition of the amino acids, is brought to 1 kg. by the addition of 632.6 gm. of D-glucose.

‡Compounds in this column, except for choline·HCl, are mixed with 48.7 gm. of D-glucose and ground thoroughly with the aid of a ball mill to yield a homogeneous yellow powder ("vitamin mixture").

§These compounds are dissolved in distilled water and the solution diluted to a volume of 100 ml. ("gluconate solution").

‖These vitamins are dissolved in absolute ethanol to give 1 ml. of solution ("alcoholic solution of fat-soluble vitamins").

¶These compounds are dissolved in distilled water and the solution diluted to a volume of 50 ml. ("acetate solution").

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1.4 gm. ethyl L-cysteinate·HCl, 60.2 gm. monosodium L-glutamate, 4.8 gm. glycine, 29.9 gm. L-proline, 15.5 gm. L-serine, and 19.8 gm. ethyl L-tyrosinate], 4.0 gm. ethyl linoleate, and 6.0 gm. Polysorbate 80. One kg. of solid dietary components is diluted to a volume of 2 liters with distilled water, as described later, and contains a total of 25 gm. of amino acid nitrogen and close to 4,000 calories—or about 2 calories per ml. This diet is isonitrogenous and essentially isocaloric with diet #26.

Diets #74, #75, and #76.—These diets differ from diet #86 only in their content of ethyl linoleate and/or Polysorbate 80. The first-mentioned diet contains 2.0 gm. ethyl linoleate and 6.0 gm. Polysorbate 80, the second, 4.0 gm. ethyl linoleate and 12.0 gm. Polysorbate 80 and, the third, 6.0 gm. Polysorbate 80 but no ethyl linoleate.

For the incorporation of ethyl linoleate and the fat-soluble vitamins into the aqueous diet phase, the following manipulations obtain. Small portions (approx. 5 ml. each) of the clear, aqueous diet solution are added, with thorough stirring, to a mixture of the Polysorbate 80 and ethyl linoleate components, which had been previously weighed into a tared beaker and treated with an alcoholic solution of the fat-soluble vitamins (table 1). After some 3 or 4 successive additions, a transparent oil-in-water emulsion concentrate is obtained that can be uniformly dispersed into the remainder of the aqueous phase. Diets prepared in this manner are of a golden hue and are crystal-clear. The phases show no inclination to separate, and the fatty components do not tend to become rancid under the conditions of normal use. Indeed, the complete diet solutions can be frozen solid in polyethylene bottles and, after storage in this state at −20° C. for at least a month, exhibit no apparent signs of deterioration.

Growth Studies

The animals employed were male, Sprague-Dawley weanling rats (N.I.H. stock) whose weights ranged from 45 to 50 gm. These animals, whose parents had hitherto been maintained on a Purina laboratory chow diet, were divided into 4 separate groups of 6 animals each, and each was housed in a separate suspension-type cage with a wire-cloth bottom. Diet solutions and water were offered to the animals *ad libitum* through glass drinking tubes, and the individual groups were provided with diets #26, #74, #75, and #76, respectively, for 82 days, after which time all groups were switched to diet #86 for 17 days. Dietary intake and weight changes were measured twice weekly, at which time the drinking tubes were cleaned and fresh diet was supplied.

The manipulations involved in the preparation of each of the diets as 50 percent solutions in water follow.[7] For each kilogram of diet (dry

[7] Directions are given for the preparation of 2 liters of diet (1 kg. on a dry-weight basis), though it is generally more convenient to prepare several times this amount for economy of time and accuracy of the measurements involved. Solutions of the "gluconates" and "acetates" (*see* table 1) may be prepared in amounts that will suffice for several weeks, and these may be safely stored for prolonged periods in the cold until required. The vitamin mixture (table 1) may also be prepared in large amounts, stored in an airtight container, and portions withdrawn as needed.

weight) that is to be prepared, 1 liter of distilled water is warmed to 70° C. and each of the essential amino acids dissolved therein with stirring. The solution is permitted to cool to room temperature, after which time the glucose component is added in small portions and rapidly stirred in order to prevent the formation of difficultly soluble lumps. The nonessential amino acids are then added, except for ethyl L-cysteinate, again with stirring until solution is complete. The water-soluble vitamin and mineral components (table 1) are added next, and the more labile components, *i.e.*, vitamins, ethyl-L-cysteinate, monocalcium fructose-1:6-diphosphate, complete the solution. Subsequently, the completed solution is diluted to volume with distilled water (2 liters of solution per kg. of diet). Although a crystal-clear solution is generally obtained at this point, the diet solution is routinely passed through a fluted filter paper in order to remove trace amounts of insoluble matter.

RESULTS AND DISCUSSION

Earlier it was demonstrated (*1*) that diet #26, which was composed of an aqueous phase (a 50 % solution in water of crystalline amino acids, the water-soluble vitamins, the requisite minerals and glucose) and a fatty-acid supplement (a solution of the fat-soluble vitamins in Mazola oil), could be satisfactorily used as the dietary regimen in long-term nutrition studies, including those bearing on growth, reproduction, lactation, and longevity. Hence, efforts to prepare a single liquid diet that was nutritionally complete in itself centered about an attempt to uniformly disperse the fat supplement of diet #26 into the concentrated aqueous phase with the aid of various partial ester emulsifiers of the Tween and Span type; such emulsifiers had hitherto been shown (*10–13*) to induce no marked untoward physiological responses when present in moderate concentration (less than 10%) in the diet of the rat. These initial efforts proved singularly unsuccessful, since every attempt to uniformly distribute the Mazola-oil component throughout the aqueous phase with the aid of a Tween,[8] a Span,[8] or a combination thereof, either required the presence of the emulsifying agent in an amount in excess of that which the animals could be expected to safely tolerate or led to the formation of an emulsion which was turbid or from which the lipide phase tended to separate after brief storage at room temperature.[9] However, like experiments, in which ethyl linoleate was used in lieu of Mazola oil, showed considerably more promise. Not only was appreciably less emulsifier required to achieve a crystal-clear emulsion with the

[8] The Tweens and Spans used in the present study included polyoxyethylene (20) sorbitan monolaurate (Tween 20), polyoxyethylene (4) sorbitan monolaurate (Tween 21), polyoxyethylene (20) sorbitan monopalmitate (Tween 40), polyoxyethylene (20) sorbitan monostearate (Tween 60), polyoxyethylene (4) sorbitan monostearate (Tween 61), polyoxyethylene (20) sorbitan tristearate (Tween 65), polyoxyethylene (20) sorbitan monoöleate (Tween 80), polyoxyethylene (5) sorbitan monoöleate (Tween 81), polyoxyethylene (20) sorbitan trioleate (Tween 85), and sorbitan monoöleate (Span 80).

[9] Somewhat more stable but still generally unsuitable emulsions could be obtained with the aid of a Minisonic Mark II sonic emulsifier.

desired amount of essential fatty-acid and fat-soluble vitamins but also the resulting emulsion could be stored at room temperature for prolonged periods, or could even be frozen at −20° C. for over a month, without apparent deterioration or separation of the phases and without developing the odor so characteristic of rancid fat;[10] for the purpose at hand, Polysorbate 80 proved to be the emulsifier of choice.

Before a nutritionally complete, chemically defined liquid diet could be employed in quantitative nutritional studies with any measure of reliability, it was necessary to establish, first, that the level of emulsifier in the diet was such as to induce no adverse physiological response in experimental animals and, second, that the concentration of essential fatty-acid and fat-soluble components in the aqueous phase would be compatible with normal growth and well-being. Thus, 3 diets were formulated, namely, diets #74, #75, and #76, wherein the constitution of the aqueous phase was patterned exactly after that of diet #26, but also contained 6.0 gm. Polysorbate 80 plus 2.0 gm. ethyl linoleate, 12.0 gm. Polysorbate 80 plus 4.0 gm. ethyl linoleate, and 6.0 gm. Polysorbate 80 but no ethyl linoleate, respectively, per 2 liters of the aqueous 50 percent diet solution.[11] In the former 2 diets, the amount of emulsifier employed greatly exceeded that required to achieve a stable emulsion with the fat and fat-soluble vitamins, while the amount of ethyl linoleate employed was designed to supply approximately 20 to 35 mg. and 40 to 70 mg. per day per rat, respectively, in the growth studies described; these latter figures stand in marked contrast to the equivalent of about 165 mg. of linoleic acid [12] per day provided to animals by the fat supplement of diet #26.

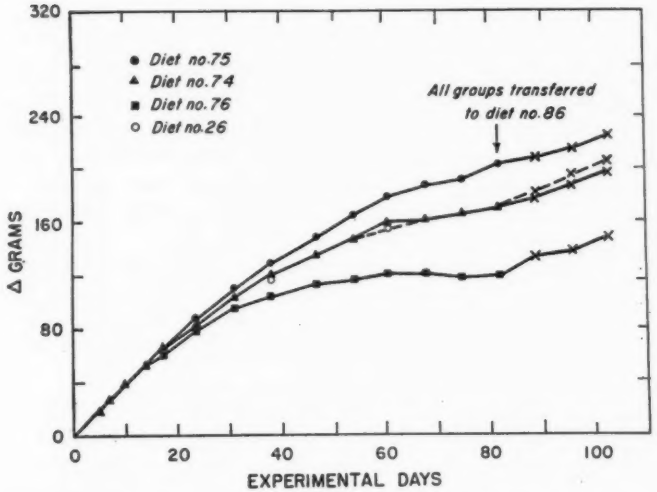
The efficacy of diets #74, #75, and #76 to maintain and promote the growth of rats was compared with that of diet #26 as follows: Male, Sprague-Dawley weanling rats (N.I.H. stock) were supplied the aqueous phase of soluble diet #26 *ad libitum* for 3 days. No essential fat or fat-soluble vitamins were provided. The animals were then divided into 4 groups of 6 rats each; at this point, the weight of the animals in each group averaged from 54 to 55 gm. One group was allowed to continue on the aqueous phase of diet #26 for an additional 82 days, but was now also supplied the customary triweekly supplement of the fat-soluble vitamins in 0.7 ml. of Mazola oil (*1*). Each of the other 3 groups was fed the single-phase diets #74, #75, and #76, respectively, over this same period. The growth response of each group of animals is depicted in text-figure 1.

Examination of text-figure 1 reveals that certain striking differences in growth response were manifested by the various groups. Thus, although the growth rate exhibited by all 4 groups of animals was very nearly the same over the course of the first 2 weeks, differences between certain of

[10] The ethyl linoleate is presumably protected from rapid autoxidation by the reductive action of certain compo, nents, *e.g.*, ascorbic acid, present in the aqueous medium.

[11] Each of the 3 diets also contained 1.0 ml. of an alcoholic solution of the fat-soluble vitamins (table 1) per 2 liters of aqueous solution.

[12] Calculated on the assumption that this component constitutes 56.2 percent of the glycerides (98.1%) of Mazola oil, according to data kindly supplied by N. E. Artz, Corn Products Co., Argo, Ill.



TEXT-FIGURE 1.—Growth response of animals maintained on chemically defined liquid diets containing varying amounts of essential fatty acid.

the groups began to emerge shortly thereafter. While a good, but virtually identical, growth rate was revealed by the groups provided diet #74 (2 gm. of ethyl linoleate per 2 liters of diet solution) and diet #26 (separate fat supplement) over the entire 82-day period, the rate of growth of the group supplied with diet #75 (4 gm. of ethyl linoleate per 2 liters of diet solution) was somewhat better. However, the growth rate of the group maintained on diet #76, which was devoid of any source of essential fatty acid but otherwise identical with diet #75, began to fall off markedly by the 30th day and by the 60th day had reached a definite plateau.

Other differences between groups, aside from growth response, became increasingly apparent during the course of the study. The group fed diet #76 suffered a progressively more severe essential fatty-acid deficiency which, at the end of the 82-day feeding period, was evidenced by a pronounced scaliness of the tail and hind legs in 5 of the 6 animals and a definite cachexia in 4. Three of the animals supplied with diet #74 revealed a mild scaliness of the tail and 2 exhibited signs of cachexia. A slight diarrhea of a transitory nature was observed in 4 of the animals maintained on diet #75. This diet contained the highest concentration of Polysorbate 80 (12 gm. per 2 liters of diet) of all the diets studied, but no perianal irritation was detected; diarrhea was previously observed during feeding experiments in which Polysorbate 80 was incorporated into conventional diets (*10–13*), but only in cases in which the emulsifier was present at relatively high levels (10 to 20%). However, none of the animals provided with diet #75 displayed any overt symptoms of nutritional insufficiency and, indeed, all appeared equivalent in health, vigor, and general physical appearance, to the animals maintained on diet #26.

With an eye toward the satisfactory growth response shown by the group fed diet #75, but also bearing in mind the occasional diarrhea induced thereby, we replaced the diets of all 4 groups of animals, after the aforementioned 82-day experimental period. The new diet mixture (diet #86) was identical in every respect to diet #75 except that it incorporated only half as much Polysorbate 80 as the latter did. The desired effect was immediate. During the 17-day period that the new regimen was provided to animals formerly fed diet #75, growth continued uninterrupted (*see* text-fig. 1), but no further sign of diarrhea was in evidence. More important, the growth response of the 2 groups of animals formerly maintained on the essential fat-deficient regimens (diets #74 and #76) was markedly improved (*see* text-fig. 1) and the cachexia and scaliness of the appendages suffered by individual animals within these groups were quite definitely alleviated.[13]

In conclusion, a single, chemically defined liquid diet has been devised that will permit rats to grow at least as well as they will on equivalent diets separately supplemented with the required amount of essential fat. This diet incorporates sufficient ethyl linoleate in clear, stable emulsion to prevent the development or to alleviate the symptoms of an essential fatty-acid deficiency. Whether the essential fat content of this diet will also suffice to meet the requirements of animals subjected to the severe stresses imposed by pregnancy and lactation cannot at present be stated.

REFERENCES

(*1*) GREENSTEIN, J. P., BIRNBAUM, S. M., WINITZ, M., and OTEY, M. C.: Quantitative nutritional studies with water-soluble, chemically defined diets. I. Growth, reproduction and lactation in rats. Arch. Biochem. 72: 396–416, 1957.

(*2*) BIRNBAUM, S. M., GREENSTEIN, J. P., and WINITZ, M.: Quantitative nutritional studies with water-soluble, chemically defined diets. II. Nitrogen balance and metabolism. Arch. Biochem. 72: 417–427, 1957.

(*3*) BIRNBAUM, S. M., WINITZ, M., and GREENSTEIN, J. P.: Quantitative nutritional studies with water-soluble, chemically defined diets. III. Individual amino acids as sources of "non-essential" nitrogen. Arch. Biochem. 72: 428–436, 1957.

(*4*) WINITZ, M., BIRNBAUM, S. M., and GREENSTEIN, J. P.: Quantitative nutritional studies with water-soluble, chemically defined diets. IV. Influence of various carbohydrates on growth, with special reference to D-glucosamine. Arch. Biochem. 72: 437–447, 1957.

(*5*) WINITZ, M., GREENSTEIN, J. P., and BIRNBAUM, S. M.: Quantitative nutritional studies with water-soluble, chemically defined diets. V. Role of the isomeric arginines in growth. Arch. Biochem. 72: 448–456, 1957.

(*6*) BIRNBAUM, S. M., GREENSTEIN, M. E., WINITZ, M., and GREENSTEIN, J. P.: Quantitative nutritional studies with water-soluble, chemically defined diets. VI. Growth studies on mice. Arch. Biochem. 78: 245–247, 1958.

[13] Close to the end of the experimental period, one animal (diet #74 group) died as the result of a respiratory infection, while another animal (diet #76 group) exhibited a lack of movement coordination, with an autopsy revealing a hemorrhagic condition in the lungs and additional damage to the spleen and liver. The weights of these animals were therefore omitted from the average growth rates plotted in text-figure 1.

(7) SUGIMURA, T., BIRNBAUM, S. M., WINITZ, M., and GREENSTEIN, J. P.: Quantitative nutritional studies with water-soluble, chemically defined diets. VII. Nitrogen balance in normal and tumor-bearing rats following forced feeding. Arch. Biochem. 81: 439–447, 1959.

(8) ———: Quantitative nutritional studies with water-soluble, chemically defined diets. VIII. The forced feeding of diets each lacking in one essential amino acid. Arch. Biochem. 81: 448–455, 1959.

(9) ———: Quantitative nutritional studies with water-soluble, chemically defined diets. IX. Further studies on D-glucosamine-containing diets. Arch. Biochem. 83: 521–527, 1959.

(10) OSER, B. L., and OSER, M.: Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. I. General plan and procedures; growth and food utilization. J. Nutrition 60: 367–390, 1956.

(11) ———: Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. II. Reproduction and lactation. J. Nutrition 60: 489–505, 1956.

(12) ———: Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. III. Clinical and metabolic observations. J. Nutrition 61: 149–166, 1957.

(13) ———: Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. IV. Mortality and post-mortem pathology; general conclusions. J. Nutrition 61: 235–252, 1957.

A Reconsideration of the Nature of the Neoplastic Reaction in the Light of Recent Advances in Cancer Research [1]

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SUMMARY

During recent years the viewpoint has become prevalent that neoplasia is a type of tissue reaction which can be provoked by various types of appropriate stimuli, *e.g.*, hormonal, other chemical, physical, and viral. This report deals with the comparative biology of cellular responses to subdamaging, injurious (irritating) agents, in general, and presents the viewpoint that dependent neoplasia may represent a special case of the well-known phenomenon of reaction-to-injury. Current theories of cancer are discussed in relation to autonomous neoplasia.—J. Nat. Cancer Inst. 24: 221–251, 1960.

THIS SPECIAL issue of the *Journal of the National Cancer Institute,* honoring the memory of Dr. Jesse P. Greenstein, comes at a historical moment in cancer research when there has been achieved "a meeting of ways" among the different disciplinary approaches to the most challenging medico-biological problem of our time—the elucidation of the nature of the phenomenon of neoplasia. As is testified by this memorial issue, Dr. Greenstein was one of the most prolific contributors to the advancement of knowledge in the biochemistry of cancer, and one of the foremost leaders of thought in this fundamental area of cancer research (*1*). In view of the meeting of ways (*2*), and the now obvious necessity of establishing interdisciplinary approaches to many of the problems that remain to be solved at the cellular and intracellular levels, it seems appropriate to include in this primarily biochemically oriented issue of the *Journal* a discussion of some of the basic biological phenomena which reflect the even more basic reactions of neoplasia at the chemical and physicochemical level.

In addition to the immediate stimulus provided by the occasion of contributing to this memorial, two other factors have prompted the author, at this time, to undertake an analytical discussion of some of the general biological manifestations of the reaction of neoplasia: First, the rapid advancement in recent years of the field of tumor virology,

[1] Received for publication July 29, 1959.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

and the resultant need for reconsidering the possibility that at least some of the virus-induced lesions, now classified in the general category of neoplasia, may be entirely dependent on viruses as their "continuing" cause—that viruses may act as "initiators" of neoplasia is no longer questioned. Second, the task of such an analysis has been greatly facilitated during recent years by a series of comprehensive reviews and discussions of various approaches to the cancer problem by leading investigators in their respective fields (*1–26*),[3] culminating in the interdisciplinary orienting discussion of Dr. Jacob Furth,[4] published in 1959 (*2*), on "A Meeting of Ways in Cancer Research: Thoughts on the Evolution and Nature of Neoplasms." In this broad, incisive, and timely commentary, Furth has emphasized the need for further consideration of a subject that has long been of interest to the author: "Comparative biology, yet to be fully explored, offers many opportunities to study the cause, manifestation, and nature of diverse neoplasms. Most needed is knowledge of events which transcend species, thus disclosing the fundamental nature of neoplasia."

It is in the search for general biological attributes of the neoplastic reaction which transcend not only species but also type of neoplasia-inducing agent that the following re-evaluation of known biological phenomena is offered for consideration.

PRESENT CONCEPTS OF NEOPLASIA

Under the impact of progressively increasing laboratory and clinical investigations, concepts of the nature of neoplasia have undergone considerable revision and evolution during the past two decades. Prior to this time, and since the establishment of the "cellular doctrine" in pathology by Virchow, "true blastomas" had been considered to differ from normal tissues, as well as from tissues undergoing reactions to infectious or injurious agents, by a special biological property denoted by the term, *autonomy*. Autonomy represented the apparent ability of tumors to grow "largely or wholly uncontrolled by the organism which supplies their nutrition," while serving "no useful function" and "reaching no definite termination of growth" (*27*). Although the progressive quality of tumor growth was recognized as being "not invariable," and it was observed that some tumors long remained practically quiescent while others actually regressed, nevertheless the state of autonomy was regarded as essentially a discontinuous (all-or-none) property by which neoplastic tissues differed from normal, or inflammatory, tissues.

Of fundamental significance to a better understanding of neoplasia has been (1) the demonstration in recent years that not all tumors of the

[3] Many additional articles could, of course, be cited, particularly those in other languages. This is intended, not as a comprehensive review, but as a listing of some of the key sources of information used by the author in the preparation of this report. See reference (*26*) for "A List of Review Articles on Cancer Research: 1951–1955."

[4] Presidential address, American Association for Cancer Research, Philadelphia, April 1958.

type previously regarded as autonomous are "largely or wholly" beyond control by their hosts, and (2) the consequent recognition of a new class of neoplasms, including highly malignant ones, now designated as dependent or conditioned. For example, as Huggins (*8*) has shown, certain highly invasive and metastatically disseminated prostatic cancers of humans are completely dependent upon male hormone supplied by the host for their continuation and progression toward an eventual autonomous state. In some cases such cancers, including their metastases, disappeared completely after removal of the source of male hormone by castration, or after the administration of antagonistic female hormone (*8*). A similar dependency of some mammary-gland cancers of humans on female hormone likewise has been demonstrated (*8*). Numerous examples of comparable hormone-dependent tumors of laboratory animals have been reviewed by Gardner (*6*, *7*), Furth (*2*, *3*), and Hertz (*9*).

More recently, the importance of another type of dependent neoplasia, namely, that associated with viral etiological agents, has become evident in the discovery of increasing numbers of viral neoplasms of laboratory animals (*2*, *4*, *15*, *23–25*), and the confirmation (*28*) of Rous's (*22*) earlier conclusion that certain neoplastic viruses are the "continuing" causes of the lesions they induce. Some virus-dependent neoplasms are therefore but continuing viral hyperplasias of an extreme type (*2*, *4*, *29*), although, with appropriate combinations of virus-infecting dose and host-susceptibility, the biological manifestations of the virus-host interaction are indistinguishable, pathologically, from the manifestations of "true blastomas" (*i.e.*, they show the cardinal features of classical malignant neoplasia, such as rapid cell proliferation, invasion of surrounding tissues, and metastasis to distant sites) (*28*).

Thus far, no virus-dependent neoplasms have actually been demonstrated in humans. However, the search for such tumors in this species has just begun and methods have not yet been developed for demonstrating them in the face of a high species specificity of the etiological agents, which might be expected if human tumor viruses have the same general properties as the known animal tumor viruses (*15*, *25*). Although several of the animal tumor viruses can produce neoplastic disease in closely related species under special conditions, such as relatively high doses of virus in extremely young or newborn hosts, they have not yet been demonstrated to transcend broad genetic barriers.

Several factors indicate the logic, and the obligation, for an extensive effort in search of viral etiological agents of human neoplasms: (1) Because of their clinical and pathological manifestations, certain neoplastic diseases, such as the leukemias and Hodgkin's disease, have long been suspected as having a viral etiology (*14*, *23*). (2) By heterologous transplantation, particularly into the guinea-pig eye, Greene (*12*, *13*) has shown that many spontaneous human neoplasms other than those of hormone-dependent tissues are also of the dependent type. (In Greene's terminology, *dependent* tumors only survive, or grow only slowly and expansively, while *autonomous* tumors grow rapidly and invade the tissues of the heter-

ologous host.) (3) Although many human tumors can be traced to known exogenous (*e.g.*, chemical, irradiation) or endogenous (*e.g.*, hormonal) etiological agents, ". . . in the great majority of cases there is no apparent cause, although we can be certain that some carcinogen is responsible" (*30*). It is not improbable that an appreciable portion of this "great majority" of spontaneous human neoplasms may be caused by viruses. The possibility exists even that a major portion of them may be so caused, and if so, a major portion of the human "cancer problem" will be in the area of neoplastic viral disease.

Of further significance for an unraveling of the riddle of neoplasia have been the demonstrations in laboratory-animal systems: (1) that there is a continuous and imperceptible transition between hyperplasia and neoplasia of the dependent type; (2) that the great majority of spontaneous tumors of laboratory animals are initially of the dependent type; (3) that such dependent tumors "go from bad to worse," *i.e.*, *progress* in degree of malignancy (*10*); and (4) that the change from dependency to autonomy is apparently an abrupt, or discontinuous, phenomenon which suggests that the two types of neoplasia "are fundamentally different" (*2*).

In summary, the concept of neoplasia has changed from that of a single disease entity, involving a single causative factor or a common intracellular mechanism, to that of a general disease category embracing a multiplicity of diseased states. "Even better," as suggested by Boyd (*30*), "It is a disease process like inflammation . . . caused by a wide variety of agents [carcinogens] which may be physical, chemical, viral and hormonal"—also, *see* Petrov (*19*). The consideration of dependent neoplasia as a general pathological tissue reaction which occurs in response to specific types of stimuli, represents the primary purpose of this report. The evidence suggesting this interpretation is presented in the succeeding section.

DEPENDENT NEOPLASIA AS A REACTION TO INJURY (IRRITATION) AND DEFENSIVE MECHANISM AT THE CELLULAR LEVEL

As already pointed out, there is a gradual transition between the biological manifestations of simple hyperplasia and malignant neoplasia of the dependent type (*2*). Since no clear-cut line of demarcation can be drawn at any point in the transitional zone between these extreme types of proliferative lesions, and since hyperplasia is known to be an integral part of all "reactions-to-injury," it is logical to consider the possibility that the exaggerated proliferative reaction represented by dependent neoplasia may constitute a special case of reaction-to-injury [5]; that is, one involving stimuli that are not so intense as to seriously damage, or destroy, the affected cells, and that are either supplied to or replicated

[5] McCutcheon (*31*) has defined *injury* as "excessive stimulation," or as "the effect on cells or tissues of excessive stimulation."

within these cells at subdamaging intensity levels, on a continuing basis. In this case the affected cells would not be expected to release the products of injury which are formed when cells are damaged to a greater degree, and which stimulate the more complex reaction of *inflammation* characteristic of the responses to injurious agents of other types or degrees of intensity. In considering the *comparative biology* of reactions-to-injury, therefore, the cellular responses in dependent neoplasia should be compared with "primitive reactions-to-injury" characteristic of lower forms as well as of the individual cells of the various types that are involved, conjointly, in complex inflammatory processes in higher forms of living animals.

Comparative Cellular Responses in Reactions to Injury

The chief phenomena of interest in this section of the discussion are two basic biological responses of cells that are primarily involved in the neoplastic reaction, namely, *proliferation* and *migration*. Irritability, or the ability to respond to stimuli, constitutes one of the primary functional attributes of living cells. As has been known since the pioneer studies of Metchnikoff, and the publication in 1893 of his book, "Lectures on the Comparative Pathology of Inflammation" (*32*), the phenomena of proliferation and migration stand out as immediately observable biological responses of living cells to injurious (irritating) agents of various types, and in all forms of animal life from the simplest unicellular organisms (protozoa) to the most complex of the higher animals, including man.

The type, and degree, of response of living cells is, of course, a function of the concentration (or intensity) of the irritating agent. Concentrations that are too high may kill the cells forthwith, or "paralyze" them so that they can show no further immediate response to the injurious agent. At the other extreme, normal constituents of cells, or of their environmental milieu, are physiologically active in the adjustment and survival of cells by stimulating functional activities, including cell proliferation and migration, within physiological limits. Excessive concentrations of such normal constitutents (*e.g.*, certain hormones and other chemical agents) may, however, produce extensive cellular damage, or "paralysis." The relative concentrations with which this discussion is concerned are intermediate between the foregoing extremes; that is, greater than the physiological levels but not so high as to produce overt cell damage, or to paralyze the primary responsive reactions of proliferation and migration. Such stimuli may be transient, in which case the lesions they produce are considered as hyperplasia and/or "repair." When the stimuli persist for longer periods they may lead to excessive hyperplasia, sometimes referred to as an "overshooting" of the repair process. Finally, if the appropriate level of irritating agent is provided on a continuing basis (*e.g.*, through hormonal dyscrasias; intracellular replication of viruses of certain types), the ultimate result may be that of neoplasia of the dependent type, or, as it has been regarded by some, "abortive repair."

Primitive Reactions to Injury

The lowest forms of animal life in which the phenomena under consideration can be readily recognized as part of a reaction to injury are the invertebrates. The earthworm, for example, has a closed circulatory system, but the latter does not take part in an accompanying "inflammatory" reaction in response to injury. After injury to the body wall of the earthworm, cells lining the body cavity proliferate and migrate toward the injured tissue. Other involved tissues, such as connective tissue and capillary endothelium, also regenerate, the blood vessels growing into the newly formed tissues during the "repair" (*31*). It is noteworthy that in the over-all "repair" process which follows simple injury, three different types of cells are involved—epithelium, connective tissue, and endothelium—and that the end-result, "repair," is accomplished through only two elementary responses (proliferation and migration) of the cells of the separate types. In the example cited, there is no stage of the reaction-to-injury associated with removal of an injurious agent, *i.e.*, "inflammation," and all of the elements of "repair" proceed immediately and simultaneously. This complex reaction may therefore be looked on as a primary response to irritation of individual cells of the various types, proceeding simultaneously. That such conjoint reactions are not necessarily "directed toward repair" but simply represent primitive defensive mechanisms of individual cells, which nevertheless result in "repair," is considered in a subsequent section.

Undoubtedly single-cell organisms, *e.g.*, protozoa, react through these same two basic mechanisms in response to mild injury. Such a pattern of reaction would be more difficult to establish among dispersed unicellular forms, however, because free-living cells normally proliferate and migrate. Evidence of a primitive defensive reaction to injury is seen, first, in the fact that when a protozoan is severed without injury to its nucleus, it will "regenerate" the lost cytoplasm through growth (*31*). In addition many chemical substances, in appropriate concentration, will stimulate proliferation of unicellular forms, but higher concentrations may paralyze or kill. Such increased proliferative activity of unicellular forms may therefore be regarded as a nonspecific primary response to irritation, or mild injury.

With regard to the phenomenon of migration, the ability of certain agents to modify the usual random pattern of normal migration of unicellular organisms is well known, *e.g.*, in the phenomenon of chemotaxis (*31*). Chemotaxis may be either positive or negative; that is, either toward or away from an agent, or level thereof, in a concentration gradient. In the absence of physical obstruction the migration associated with chemotaxis is in a straight line (*31*), but if obstructions exist it might be expected to follow the least line of resistance. Since migration appears to represent a primary response to irritation, this reaction might be expected to be activated also by irritating agents which gain entry into single-cell organisms, such as ingested harmful bacteria which cannot be readily digested, viral agents of certain types, or chemical substances which

because of their properties accumulate selectively within the cells. Actual evidence of such stimulation is seen in higher forms of animals in which "free-living" phagocytic cells, through this primary reaction, sometimes activate the dissemination of certain disease-causing agents throughout an organism (*see* subsequent section).

Cellular Responses in Complex Reactions to Injury (Inflammation and Repair) in Higher Animals

The pathological observations and concepts reiterated in this section are taken from standard textbooks on human pathology (*30–37*). Although most of the information can be found in any of the separate sources, specific references are cited where quotations are used or where the statements approach direct quotations.

"It is difficult to draw a hard and fast line between repair and inflammation. Both represent the reaction of tissues to an irritant"(*30*). In its restricted sense, the term *inflammation* applies to the local reaction of the body to irritation, featured by vasodilation, increased capillary permeability, and the emigration of leukocytes to the injured area. With accompanying apologies for use of the teleological term, the "object" of this phase of the reaction is said (*30*) to be "to destroy and remove the irritant." This is accomplished chiefly by the leukocytic elements, whose emigration is facilitated by the vascular reactions. *Repair* includes both the regeneration of tissues of the type destroyed, as well as their replacement by other tissue, *e.g.*, fibrous, where the damaged cells are not capable of regeneration. In both cases repair is accomplished through proliferation and migration of one or more types of cells in response to irritation. The "object" of this phase of the reaction is said (*30*) to be "to repair the damage done to the tissues." As a rule, but not always, inflammation precedes repair (*30*). This is particularly true where bacterial infections are involved, and where extensive local as well as systemic reactions occur as a result of the interaction between host tissues and invading organisms. In the absence of infection, "repair processes may go on at the same time as inflammation" (*30*).

Reactions in the absence of infectious agents.—When a clean incised wound such as a closed surgical incision heals "by first intention," there may be only a slight inflammatory reaction and the events do not differ very markedly from the primitive response-to-injury described in the preceding section. There is some vasodilation, a small amount of exudate formation, and limited leukocytic infiltration, but the "repair" process starts within a few hours in such wounds, apparently as a primary response-to-injury. Connective-tissue fibrocytes and capillary endothelial cells are the first to undergo proliferation and migration. This results in a reuniting of the edges of the wound and a restoration of the severed blood supply. Later, the epithelial cells of the skin undergo proliferation and migration, resulting in closure of the epithelial gap.

"It might be thought that the new epithelial cells were produced by mitosis. This is not the case. The defect is made good by the migration

or sliding of cells from the edges of the wound. Mitoses are found regularly at some distance from the edge" (*30*). The migration of epidermal cells in sheets, in contrast to fibrocytes which migrate separately, is because the cells of the epidermis are firmly attached to each other through intercellular bridges. The mechanism of locomotion of the sheets appears to depend on ameboid motion of the constituent cells (*31*). In the closing of small wounds of the skin, as in wounds of the cornea limited to the epithelial layer, the latter cells "move in from the sides and may close the defect in as short a time as six hours. Closure is here accomplished solely by cell migration; cell division plays no part" (*31*). In the closure of open wounds such as those involving abrasions of the epidermis, or open ulcers, the phenomenon of cell proliferation predominates. The first cells to become involved are the fibrocytes and vascular endothelial cells, which, by proliferating and migrating together, result in the formation of "granulation tissue." On this newly formed mesenchymal base, epithelial cells move in from the edges of the wound by migration, aided by cell division. "Cell division may continue after migration has ceased with the closure of the wound, until all the cells lost have been replaced" (*31*). "New tissue is formed hurriedly and in great quantities to replace that which is lost, and although at first it seems to grow in a somewhat disorderly way, it quickly shows an adaptation to its purpose" (*35*). If for some reason healing is long delayed, as in a chronic ulcer, "the epithelium sends long processes down into the deeper tissues. These may appear to be detached from the surface in microscopic section, and may be very suggestive of carcinoma. In some cases a malignant growth may actually commence in such a chronic ulcer" (*30*).

It seems evident from the foregoing excerpts from modern descriptions of pathological processes, that *proliferation* and *migration* are not, in themselves, to be regarded as pathological manifestations of cells. Rather, they should be regarded as two of the most basic reactive properties of living cells in general, which take part in normal cell function and survival under certain conditions, and which also may become activated to an exaggerated degree when injurious agents are encountered. Such exaggerated activity is related to the strength, and duration, of the abnormal stimuli. In complex multicellular organisms of the highest order, many cells of the body become highly differentiated and do not normally show appreciable proliferative or migratory activities. When subjected to suitable stimuli, however, these latent primitive reactive potentialities may manifest themselves, at least to some degree, in all but the most highly differentiated types of cells which have entirely lost these potential powers, *e.g.*, parenchymal cells of the central nervous system. Further evidence that differentiated cells of complex tissues retain the primitive properties of proliferation and migration is found in the well-known fact that both of these reactions are manifested *in vitro* after the cells have been carried through several passages in tissue culture. Clearly, dependent neoplasia could result from the continued activation of these basic responses by just the right kinds and concentrations of irritating agents.

Reactions in the presence of infectious agents.—An attempt to include in this discussion the varied pathological pictures which involve the phenomena of proliferation and migration in interactions of host cells with the numerous known disease-producing organisms would be but to reiterate large segments of textbooks on pathology; for, the history of inflammation, as Boyd has said, "is the history of pathology" (*30*). The discussion will therefore be restricted to two examples of inflammatory reactions in which the overgrowth of tissue may under certain conditions approach the picture of neoplasia.

The first involves a highly resistant organism, the tubercle bacillus. Tuberculosis belongs to the group of chronic inflammatory diseases known as the *infectious granulomata*, which is actually a misnomer since the characteristic proliferative lesions are composed not of granulation tissue, *i.e.*, fibroblasts and vascular endothelium, but of emigrant leukocytes (monocytes and macrophages) which themselves undergo extensive proliferation and migration. The variations in pathological manifestations of tuberculosis are multitudinous, and are a function both of the degree of resistance or immunity of the host and of the dose and virulence of the infectious organisms.

When a highly susceptible host is infected for the first time with the most virulent strains of tubercle bacilli, the first inflammatory cells to emigrate to the injured area are the polymorphonuclear leukocytes. "They are actively phagocytic, but are unable to damage the bacteria which they engulf" (*30*). This is due to the waxy protective coat of the bacilli. Later, large mononuclear cells are attracted to the area through chemotaxis, which also can be stimulated by the waxy constituent, alone, extracted from the bacteria. The mononuclears are highly phagocytic members of the reticuloendothelial system which engulf not only the free bacteria but also the polymorphonuclear cells which have phagocytized such organisms. The mononuclears are more successful in handling the bacilli, but cannot digest them completely. They end up with a dispersion of the bacterial lipide throughout their cytoplasm, and are somewhat damaged in the process. Such cells have a typical morphologic appearance and represent the "epithelioid cell," which is the most characteristic feature of the tubercular reaction. "Giant cells," also typical, are formed by the fusion of a number of epithelioid cells. "The small mass of newly formed or newly arrived cells constitutes a tiny translucent nodule visible to the naked eye and known as a *tubercle* or *miliary tubercle*." The latter "increases in size and several tubercles may fuse to form a larger mass" (*30*). In some cases there may be spread of the infection by migration, "the bacilli being carried by phagocytes through the tissues, giving rise to the formation of new tubercles which fuse together until large caseous areas are formed" (*30*). Thus, activation of the primary reaction of migration, evidently by a subdamaging intracellular dose of tubercle bacilli, may be an important factor in the dissemination of the disease by phagocytic cellular elements whose "purpose," supposedly, is that of destroying and removing the offending agent.

Under other conditions described by Boyd (*30*), in which the mononuclears do not have the power of destroying the bacilli which they engulf, the reaction of proliferation may become activated to the extent that the end-result may be entirely analogous, biologically, to dependent neoplasia. "Thus in the rat the bacilli thrive and multiply within the phagocytes, and the latter also multiply with the remorselessness of a malignant growth, until finally the animal succumbs to the cellular accumulation" (*30*).

The second example of a bacterial proliferative lesion with "definitely neoplastic features" (*33*) is verruga peruana, or a mild form of Carrión's disease, which occurs in relatively immune individuals as a result of infection with *Bartonella bacilliformis*. In immune human hosts the infectious organisms are present in large numbers in the reticuloendothelial cells throughout the body, and multiple cherry-red cutaneous nodules appear on the face and extremities. "Microscopically the picture resembles that of a rapidly growing capillary hemangioma, with numerous mitotic figures" (*33*). The proliferative lesion is thus a result of stimulation of endothelial cells by an intracellular, bacterial, irritating agent. It is significant that this same intracellular parasitic organism produces a rapidly progressing febrile anemia (Oroya fever), which often is fatal, in subjects who have little or no immunity to it (*33*). The lesser damaging effects of the intracellular bacteria in partially immune hosts, and the activation of the cellular proliferative reaction under the condition of this relative state of agent-host balance, is comparable to the set of circumstances described by Duran-Reynals (*15*) for the Rous sarcoma virus in chickens of different ages, and used by him as a basis for his hypothesis that, fundamentally, neoplasia is a reaction to viral etiological agents of hosts possessing a certain degree of resistance to such agents. Although this relative state of agent-host interaction is undoubtedly the underlying factor in the activation of many dependent neoplasms, it cannot be the universal "cause" of all such neoplasms.

Perhaps it would not be illogical to admit to the category of dependent neoplasias this group of bacteria-dependent, excessive, proliferative lesions.

Proliferation and Migration as Primitive Defensive Reactions at the Cellular Level

It has already been established that both proliferation and migration occur as primary cellular reactions to many types of stimuli. Why they occur, as basic processes of living protoplasm, is not a question for present consideration, but one which involves more fundamental life phenomena at the biochemical and physicochemical level. It is the purpose here simply to consider the question: Since these responses do occur in reaction to irritation, under what conditions would they tend to reduce the intensity of the stimulation that provokes them, and therefore constitute, incidentally, primitive defensive reactions at the cellular level?

Proliferation

A condition under which cell proliferation might reduce the intensity of stimulation would be one in which the offending agent were either concentrated selectively or replicated within cells at a rate less than that at which the cells were capable of building up new protoplasm. That cell division accompanies growth in the proliferative reaction is incidental to the present discussion. The increase in protoplasmic mass through expenditure of available energy supplies would tend to dilute the agent as it reached excessively stimulating levels. If the supply of agent were inexhaustible, relative to the time required for the production of large masses of tissue, the continuation of the reaction, although beneficial for survival of the affected cells, could result in neoplastic overgrowths of tissue detrimental to survival of the organism as a whole.

Those familiar with the earlier literature will recognize that many of the foregoing comments represent simply a different way of saying, with a sort of physiological slant, exactly the same things that were said by Rous (*22*) in 1943, or earlier. Of interest, therefore, are Rous's description and discussion of certain *near-neoplastic* reactions which in the present connection might be considered as examples of the condition in which the supply of offending agent is not inexhaustible, but limited:

> Pathologists have realized for a long time that normal cells while functioning may behave for a brief while as if neoplastic, those of the chorion, for example. In 1906 it was found that the intradermal injection of oily solutions of certain dyes, notably scharlach R and sudan III, was frequently followed by local downgrowths of the epidermis in narrow strands which penetrated amid the corium and sometimes entered lymph vessels, giving a picture which wholly resembled anaplastic, squamous cell carcinomatosis, except for the droplets of stained oil in the tissue (*38*). The malignant activities continued only so long though, as dye was present, the epidermis eventually forming an orderly layer around the decolorized oil: the stimulus experimentally provided had run out, but a further temporary downgrowth could be obtained by repeating the injection. These phenomena not only made it plain that ordinary cells are able to behave as if cancerous in response to extraneous influences but they justify the inference—though this was not made at the time—that an actuating cause for tumors need only work on capabilities which cells innately possess, and go along with them as they multiply in response to its presence.

Only two comments need to be added to this quotation from Rous to bring it up to date: (1) At the present time, his statements are definitely known to apply only to those neoplasms now classified as *dependent*. (2) In addition to the viral agents implicated by Rous, which replicate within living, functioning cells, agents of other types likewise are capable of eliciting the whole spectrum of proliferative lesions from mild hyperplasia and near-neoplastic reactions to highly malignant neoplasias of the dependent type. These agents are the hormones, which, like the fat-soluble dyes discussed by Rous, provoke neoplastic reactions when supplied on a continuing basis. The "cause" of the continuation may be a natural hormonal dyscrasia (*8*), or it may reside in artificial laboratory

procedures such as the repeated administration of specific hormones in doses above physiological levels; the continuation may also be brought about by experimental alterations in normal regulatory feed-back mechanisms, which result in the removal of physiologically antagonistic hormones (*2, 4, 6–9*).

It is evident from the foregoing considerations that the reaction of cell proliferation can, under special circumstances, serve to retard the build-up within cellular protoplasm of irritating agents which have a selective affinity for, or which replicate within, living cells. In this sense the phenomenon of proliferation may be regarded as a primitive, or cellular, defensive mechanism.

Migration

That migration can remove free-living unicellular organisms from unfavorable environmental factors is obvious and has long been recognized in the phenomenon of negative chemotaxis (*31*). It is possible that the "wandering" phagocyte cells of higher organisms can likewise benefit, as individuals, by this primitive defensive reaction. An example seems apparent in the centrifugal migration of some of the mononuclear cells from areas of high concentrations of tubercle bacilli noted in a previous section.

It is logical to assume that the phenomenon of migration, being a basic reaction of living protoplasm to stimulation, might be activated, also, by unfavorable concentrations of an agent built up within individual cells as a result of agent replication, or through its selective affinity for the particular cells. Then the migration would not be expected to be unidirectional, as in negative chemotaxis, but random in nature, following the least line of resistance in the presence of physical obstructions. This would explain the invasion of surrounding normal tissues by neoplastic cells, as well as their metastasis to distant sites. Migration of neoplastic cells occurs only at the periphery of the tissue mass where the closely packed cells have some freedom to migrate, and in those areas of lesser physical resistance provided by the lymphatic and vascular capillaries which permeate the involved region. It is the migration into these latter channels that provides the basis for distant metastasis. This phenomenon is observed even among completely dependent neoplastic reactions as the lesions gain momentum in proliferative activity.

It is clear that "extraneous influences" acting on "capabilities which cells innately possess" (*22*) can account for the phenomenon of migration, which represents one of the primary biological characteristics of dependent malignant neoplasia.

CURRENT THEORIES OF CANCER AND THE PHENOMENON OF AUTONOMY

Is *autonomy* an ultimate limit which all neoplastic reactions approach asymptotically through continued progression, or does it represent a

"fundamentally different" (*2*) reaction resulting from a quantal transition of normal or of dependent neoplastic cells? The basic information necessary for answering this question decisively is lacking at the present time. However, a reiteration of certain available information may be of aid in attempts to discriminate among different candidate "theories of cancer," or in evaluating the possibility that more than one mechanism can lead to the same biological end-result—that of autonomous neoplasia.

The concept of complete, or practically complete, independence of tumors with respect to their hosts, *i.e.*, except for the provision of stromata and the supply of nutrient materials, arose primarily from studies on human neoplasms [*c.f.* (*27*)], before the emergence of cancer research as a scientific discipline. It was this extreme and clinically dramatic type of neoplasm that attracted the most attention and dominated the thought in formulations of theories to account for the mysterious lesions.

Biological Theories

Two of the oldest biological theories still under current consideration are: (1) the virus theory, and (2) the theory of somatic mutation. (Other current theories, involving biochemical mechanisms, will be mentioned later.) The somatic mutation theory, although chronologically newer, will be considered first, not only because it provides a basis for a discussion of certain newer aspects of the virus theory but also because it was founded on an older pathological observation which had long been considered as characteristic of highly malignant human neoplasms, *i.e.*, the frequent occurrence of abnormal, multipolar, mitoses.

Somatic Mutation Theory

The hypothesis now generally referred to as the "somatic mutation theory" (*5*, *39*) had its origin in a treatise published by Boveri (*40*), in 1912, on "The Origin of Malignant Tumors." Earlier studies by Boveri had resulted in the experimental induction of multipolar mitoses in sea-urchin eggs, both by double-fertilization and by mild injury of newly fertilized eggs through mechanical agitation. In both instances "pathological" forms were observed during the further proliferation of the fertilized egg up to the blastula stage. On these observations Boveri constructed his hypothesis that the asymmetric distribution of chromatin material in daughter cells during multipolar mitosis could give rise to pathological intracellular states, most of which might be expected to result in death of the cells but some of which, according to a certain probability, could give rise to excessive cell proliferation of the type encountered in neoplastic cells. The essence of his theory was not in the abnormal mitosis *per se*, but in the fact that such a phenomenon could give rise to "a wrong arrangement of the nucleus." Referring to the chromosomal make-up of a particular cell as its "chromatin-complex," Boveri's basic concept was that the neoplastic state was a result of "a *certain abnormal chromatin-complex, no matter how it arises.*"

Recognizing that both cell structure and cell metabolism are under chromosomal (or genic) control, he considered as "certain" the fact that ". . . histological changes go hand in hand with a changed *metabolism* of the tumor cell." The further formulation of the concept is most precisely reproduced in Boveri's own words:

> The normal products appear in different relations, or entirely different products are formed, which differ at any rate from those of the cells of the normal original tissue . . . . if an abnormal *chromosome* combination exists in the tumor cell, and also an abnormal combination of chromatin *material*, then it is clear that the metabolism of such a cell is abnormal. The tumor cell, which lacks certain chromosomes while it has too large a number of other chromosomes, will produce many substances in too great, others in too small, quantities, or none at all, and it is very probable that the material of single chromosomes which are otherwise normal so act on each other under altered quantitative relations that the products which result are very different from the normal end products.

It is thus clear that Boveri's original concept was of the nature of a reaction to injury at the chromosomal level. However, it was not the defective "chromatin-complex" *per se* which provided the immediate stimulus for abnormal cellular behavior, *i.e.*, proliferation and migration, but the stimulating (irritating) substances produced within the cells by such abnormal chromatin complexes. Since the chromosomal defect was considered to be irreversible, and heritable, a theoretical mechanism was provided for maintenance of the abnormal tissue reaction on a continuing basis, *i.e.*, neoplasia.

Among the factors which might cause the same end-types of derangements in chromatin-complexes, Boveri placed special emphasis on substances capable of depressing cell activity, which might, with a certain probability, inhibit cell division after it had been started, but before its completion. Thus, arrestment of the process just after the centrosome had divided would provide the basis for multipolar mitosis in a subsequent division. Other factors mentioned by Boveri as being capable of producing abnormal chromatin-complexes were: (*a*) Intracellular parasites [*see* succeeding discussion of the virus theory]; (*b*) external influences, which act perniciously on certain chromosomes while they do not affect others; and (*c*) certain chromosomes, which become diseased, owing to an inherited disposition. The last of these factors caught the attention of geneticists, who were the only biologists at the time to pay much attention to the theory of Boveri. It was the subsequent development along genetic lines, of the thinking with respect to a chromosomal origin of cancer, that resulted in the introduction of the concept of *somatic mutation* (*see 5, 39*), and that gave the more general theory its present, restricted name. The genetic version of the theory placed the emphasis not on chromosomal defects as a type of injury but on the mutation of genes, either spontaneously or at increased rates under the influence of mutagenic agents. Such mutations were, of course, well established as basic biological phenomena, which are active in both biological variation

and evolution. This mechanism was logically conceived since selective inbreeding had resulted in the establishment of inbred lines, particularly of mice, which developed several different types of spontaneous tumors, with very high incidence. The end-result of the somatic mutation concept, which placed the mechanism of cancer entirely within the bounds of classical genetics, was a new viewpoint toward the cancer cell itself (*5*, *39*, *41*). Thus the cancer cell came to be looked on as an altered cell, apparently normal within its own frame of reference, but different from the tissues of the host in which it arose and on which it was now, for all practical purposes, a parasite, *i.e.*, ". . . a cell with a capacity for unlimited or uncontrolled growth" (*41*). This is in contrast to Boveri's original concept, as well as to those concepts discussed in previous pages, in which the cancer cell is viewed as basically a genetically comparable cell, but one which has sustained a chromosomal injury, or is currently involved in a self-preservation struggle with a continuing injurious (irritating) agent.

The chief merit of Boveri's general theory is that it provides a wide basis for explaining the quantal transitions of cells that are known to occur in some types of proliferative reactions, *i.e.*, from the large body of evidence that has accumulated in work with carcinogenic agents. Since the postulated chromatin defects are not necessarily specific in nature, and since they can undoubtedly occur to different degrees, a mechanism is at hand for explaining the varying degrees of hyperplasia and neoplasia among cells that give evidence of having undergone a quantal transition of some type. For example, Rous and Rogers (*42*) painted localized areas of the skin of experimental rabbits with weak concentrations of carcinogenic chemicals and obtained no morphological evidence of cellular change. However, if a wound were made both through the treated area and through the adjacent normal skin, a temporary overgrowth of the epidermis occurred in the treated area during the process of repair. The cells were not malignant, and did not continue to grow after the temporary stimuli of repair had ceased; but the phenomenon did indicate quite clearly that the treated cells had undergone a quantal transition with respect to their capacity to proliferate, as a result of the influence of the carcinogenic chemicals. A similar quantal transition could logically explain the "latent" cancer cells (*43*, *44*) that are produced with weak doses of various carcinogens, but that do not reveal themselves unless they are further stimulated by some "promoting" or "co-carcinogenic" agent such as croton oil.

Another experimental neoplasia as yet unexplainable on any other basis is that induced with ultraviolet light. Tumors induced with this agent are of the dependent type, since both the time of appearance and the rate of growth of the tumors, even after discontinuation of ultraviolet treatment, are a function of the initiating dose (intensity and duration) of ultraviolet light (*45*). This would seem logically explainable by the perpetuation of quantal changes in the chromatin-complex which might occur to different degrees under the influence of different total

doses of ultraviolet light. However, as discussed by Blum (*45*, *46*), these results cannot be accounted for on a basis of somatic mutation in the genetic sense.

It is probable that reactions to injury at the chromosomal level can and do cause a spectrum of proliferative lesions from limited hyperplasia through at least dependent neoplasia. It does not involve any stretch of the imagination to extend this mechanism to the degree of chromosomal alteration that would be required to account for the extreme manifestations of autonomous tumors.

A more accurate designation of Boveri's original, general concept of "the origin of malignant tumors" would be *the chromosomal injury theory*. This, of course, does not imply that the genetic version of chromosomal participation, through *somatic mutation*, may not also be of importance in the causation of some tumors (*e.g.*, where heritable constitutional factors appear to be involved—*see* succeeding section), but merely that this phenomenon cannot account for all tumors [*cf.* (*28*, *45*)] and therefore cannot represent a universal theory for the causation of cancer.

Virus Theory

Conceptual mechanisms

The virus theory, like the theory of somatic mutation, is not a single unified concept but involves several different variations with respect to the mechanism by which viruses are supposed to induce, or activate, the reaction of neoplasia. The original concept, first advanced by Borrel (*47*) in 1903, places the virus in a "continuing" role and views neoplasia as a virus-cell interaction of the nature of an extreme viral hyperplasia. The earlier history of this concept has been reviewed by Oberling (*48*), and analytical discussions from the same point of view have been given by Rous (*22*, *49*), Andrewes (*50*), Oberling and Guérin (*51*), and Duran-Reynals (*15*).

Another concept of viruses in the causation of neoplasia, which, however, was not proposed or even embraced by adherents to the virus theory, is that viruses are merely "biological carcinogens" capable of activating certain neoplastic potentialities, which cells already possess, in much the same way as various other types of carcinogenic agents [*i.e.*, chemical and physical—*see* (*52*, *53*)].

A third version of the role of viruses in neoplasia has emerged on a basis of a new biological phenomenon that has been observed in bacteriophage—bacteria systems, namely lysogeny, or "parasitism at the genic level." In this phenomenon, viral (bacteriophage) nucleic acid becomes integrated with the nucleic acids of the host-cell chromosomes and is thereafter replicated in single copies only, along with the normal genes during subsequent cell divisions. Such parasitic or foreign "genes," designated as "prophage" in the bacteriophage systems [*see* (*54*) and (*55*) for discussions], undergo spontaneous mutations with a certain low frequency characteristic of a given system. In the bacterial cells in which such mutations have occurred, mature phage particles, in which a pro-

tective protein jacket surrounds the nucleic acid "nucleoid," appear in large numbers in the cytoplasm and bring about the eventual lysis of the involved host cells. The rate of such mutations can be increased by ultraviolet irradiation as well as by other mutagenic agents, some of which also possess tumor-inducing properties (*54*). It has been suggested (*55*) that a similar integration of the nucleic acids of tumor viruses with the genetic material of their animal host cells could account for the inherited factor associated with the development of spontaneous tumors in certain high tumor strains of inbred laboratory animals.

The viral agent which provokes a high incidence of mammary-gland carcinomas in certain inbred strains of mice (Bittner virus) (*56*) has been shown to be transmitted under natural conditions, primarily through the mother's milk. This agent is therefore extrachromosomal (*57*) and its transmission does not appear to be consistent with the foregoing hypothesis.

Two additional types of neoplasms, lymphocytic leukemia and pulmonary adenomatosis, also occur with high frequency in inbred strains of mice and yield transmission results which, under natural conditions, appear to be consistent with the theory of parasitism at the genic level. As pointed out by Law (*58*), the transmission of leukemia from generation to generation follows the pattern expected for chromosomal transmission, and no evidence has thus far been obtained of extrachromosomal transmission of the tumor-inducing factor itself in the perpetuation of the disease in inbred lines. Yet, as Gross (*59*) has shown, a filterable agent can be isolated from tissues of such spontaneously occurring leukemias, which will reproduce the disease when inoculated into recipient mice of susceptible indicator strains.

In the last of these three most prevalent spontaneous neoplasms of inbred mice, *i.e.*, pulmonary adenoma, no filterable agent has thus far been demonstrated. The long-sustained and highly fundamental studies of Heston (*60*) have shown quite convincingly that the heritable factor associated with the transmission of this disease is indeed located in the chromosomes of the sex cells. The evidence for this conclusion resides in the demonstration of linkages between the lung-tumor-inducing factor and certain other well-known marker genes which can be traced through the characteristic anatomical abnormalities with which they are associated (*e.g.*, lethal yellow, hairless, the three linked genes, shaker-2, waved-2, and vestigial tail, fused, flexed tail, and obese). It will be of interest to see whether a "meeting of ways" between genetics and virology will result in a final understanding of these, at present, baffling hereditary neopastic diseases. [For recent analytical discussions of the present status of the tumor-virus problem, *see* (*2*, *4*, *15*, *23–26*).]

The phenomenon of autonomy

In accounting for the phenomenon of autonomy on a basis of the virus theory, or theories, it may be necessary to assume that more than one mechanism can lead to the same biological end-result if the autonomous

lesions produced by different known types of tumor viruses are to be explained.

An example is seen in the different findings obtained with two of the oldest and most widely investigated tumor viruses, viruses of Rous sarcoma and Shope papilloma, which differ from each other not only in their chemical compositions but also in their intracellular locations and their specific biological manifestations.

Rous sarcoma virus.—The Rous sarcoma virus is specific for the connective-tissue cells of domestic chickens, although under certain conditions it can infect similar cells of closely related fowls. The virus particles contain nucleic acid of the ribose (RNA) type (*61*, *62*), and are characteristically found primarily in the cytoplasm of the Rous sarcoma cells (*63*, *64*).

The tumors produced by this virus are of the nature of viral proliferative lesions, dependent in all of their biological properties on the concentration of virus at a given time, *i.e.*, in relation to the level of natural host susceptibility (*28*). Tumors produced by the weakest doses of virus show considerable dependency on host factors and display wide variations in their biological characteristics, including the amount of virus that can be recovered from the tumor tissues. In some cases active virus cannot be detected in extracts of the "low dose" tumors (*28*). Tumors produced by the stronger doses converge toward a common highly malignant and autonomous type, with little variation among different hosts of a common genetic type. The virus is present in high concentrations in extracts of such "high dose" tumors (*28*). Studies of the chromosomes by newer methods have not been made on Rous sarcoma cells, but in studies of chick-embryo cells infected with the virus *in vitro*, no evidence of polypoidy or other chromosomal abnormality was observed (*65*). However, both the nuclei and nucleoli are enlarged, and margination of chromatin is seen in resting cells (*66*). It appears that in this neoplasm an overwhelming concentration of the virus itself is the factor responsible for the condition of autonomy. This is in keeping with the original version of the virus theory in which the virus is considered as the sole continuing cause, and the lesion as an extreme virus-cell interaction. This conclusion is further substantiated by the fact that, under the influence of strong doses of the virus, mature fibroblasts undergo direct conversion to neoplastic cells within 48 to 72 hours, without a preceding phase of extensive cell proliferation (*66*).

Shope papilloma virus.—The Shope papilloma virus is specific for the epidermal cells of the skin of rabbits. Although lesions are produced in both wild and domestic rabbits, which are of different genera, the virus is recoverable in appreciable quantities only from the papillomas of the former, which are the natural hosts. The particles of Shope papilloma virus contain nucleic acid of the deoxyribose (DNA) type (*67*), and are characteristically found primarily in the nuclei of the papillomatous cells (*68*).

In its initial sojourn, the virus-induced lesion represents simply a

persistent viral hyperplasia of the benign type, which may appear to remain stationary in size over periods of many months. At this stage no evidence of polypoidy or other chromosomal abnormality is found (*69*). Eventually, however, a certain percentage of the benign lesions undergo a sudden transition to the malignant state, the percentage of such transitions being apparently related to the concentration of the virus which initiates the benign lesions (*70*). The malignant tumors are typical epidermoid carcinomas which meet all of the criteria of the "true blastomas," including the fact that virus cannot be recovered from them, even in the natural hosts, wild rabbits (*53*). That many of these carcinomas are autonomous is indicated by their rapid proliferation and high degree of transplantability in noninbred hosts (*52*). The malignant derivatives, in contrast to the initial benign lesions, display chromosomal abnormalities in the form of polypoidy (*69*).

In the absence of adequate information to permit discrimination among the different possible mechanisms which could account for the malignant transformation, any of the separate versions of either the somatic mutation or the virus theories might be logically invoked as a likely candidate. For example, the intranuclear parasite (virus) might cause chromosomal misarrangements as suggested by Boveri (*see* page 234), or the more rapid cell proliferation of the benign lesions could increase the chances of an appropriate "somatic mutation." With respect to the viral hypotheses, the second of the conceptual mechanisms mentioned previously has been favored by many as the most likely cause, *i.e.*, the activation of an inherent cellular malignant potentiality by the virus, through its activity as a "biological carcinogen." Among the evidence considered as favoring this possibility is that provided by Rous and his collaborators on the synergistic action of the virus and certain carcinogenic chemicals. Thus, the transformation to malignancy, which ordinarily does not take place until many months after the initiation of the benign lesion, can be brought about within a few weeks through applications of a carcinogenic chemical to the benign lesions (*71*). The same end-result may be obtained also by the localization of intravenously injected virus into areas of skin previously treated with the chemical (*72*), or by simultaneous treatment of the skin with both the virus and the chemical (*73*). (The carcinogenic chemicals alone also require many months for the induction of neoplasms in rabbits.) Such synergistic action could, however, be as logically interpreted as an enhancement of the transition, brought about through any one of several of the other hypothetical mechanisms.

A recent fundamental discovery would appear to admit the possibility that the virus itself, in the form of viral nucleic acid, might account for the malignancy, as well as autonomy, in much the same way as the Rous sarcoma virus does, in spite of the fact that active or "mature" virus cannot be demonstrated in the malignant carcinomatous lesions. This discovery is the finding of Noyes and Mellors (*68, 74*), that antigenically and biologically active virus can be demonstrated *only* in the keratinizing papillomatous cells, which have ceased proliferating and started to undergo

differentiation. Virus is not demonstrable in the actively proliferating cells of the basal and malpighian layers of the neoplastic epidermis. These observations were interpreted to indicate that viral nucleic acid, only, is reproduced by the actively proliferating cells, and that the nucleic acid moiety constitutes the continuing cause of the benign proliferative lesions. Only when the cell itself undergoes differentiation does the process take place that results in the viral nucleic acid acquiring its protein protective jacket, which not only gives the virus its transmissible quality but also provides the antigenic specificity by which the virus can be detected (*68*). As already pointed out, active virus cannot be demonstrated in the malignant tumors, even in the natural hosts. The cells of such malignant tumors are anaplastic and show no tendency toward keratinization. If the interpretations of Noyes and Mellors, regarding the benign lesions, represent the actual situation, high concentrations of viral nucleic acid could also be the continuing cause of the autonomous malignant tumors induced by the Shope papilloma virus.

Confirmation of the conclusions of Noyes and Mellors could have a profound influence on experimental approaches to the tumor-virus problem in general. For example, it not only would make reasonable an earlier concept that neoplastic reactions may be driven by virus in a "masked" or "occult" form [*cf.* Shope (*75*) and Andrewes (*50*)], but it would also provide a basis of hope for a sound experimental approach from this point of view, which previously had appeared to lead only to a dead end. Thus: (*a*) "Occult" or "immature" virus in the form of viral nucleic acid might possibly be made to undergo maturation, *e.g.*, by appropriate measures which would slow down the proliferation and promote the differentiation of host cells, thus permitting the transmission and experimental demonstration of such virus. (*b*) Biochemical methods might possibly be devised for the isolation of such viral nucleic acids, as well as for protecting them against host enzymes during transmission and infectivity tests. This has already been achieved (*76*) in the separation of certain viral nucleic acids from their protein constituents. Such isolated nucleic acid preparations have been found to be fully capable not only of initiating the characteristic viral disease in new hosts but also of causing the cells of these hosts to manufacture "mature" virus. If comparable technical methods applicable directly to cancerous tissues could be achieved, it is possible that the isolated nucleic acids of tumor viruses might be made to cross tissue and species barriers. This possibility is suggested by the success already achieved in this respect in tissue-culture systems with the ribonucleic acids of several of the enteroviruses (*77*). Such might be the means for overcoming the species barrier associated with the testing of human tumor viruses in laboratory animals, which at the present time appears to be an almost insurmountable obstacle to this experimental approach to the human problem;

Theories Based On Biochemical Mechanisms

Two additional theories under current consideration that are founded on large bodies of experimental evidence are: (1) the Warburg theory, and (2) the deletion theory of cancer formation. Both were proposed by biochemists, and both involve conceptual mechanisms at the biochemical level. A discussion of the actual biochemical phenomena is beyond the scope of this biological analysis, as well as the area of competence of the author—*see* (*78–84*) for comprehensive discussions of the biochemical mechanisms. It is the purpose here merely to recount in a general way the basic ideas behind the theories, and to discuss their relations to known biological phenomena of neoplasia.

Warburg Theory

The Warburg theory of the causation of cancer had its origin in the discovery by Warburg (*78*) in 1923 that cancer cells have a decreased oxidative metabolism and an increased fermentative metabolism as compared with normal growing cells. This basic observation has stood the test of time, and a large amount of experimental evidence has accumulated to show that the ratio of glycolysis to respiration in the production of energy is quantitatively related to the degree of malignancy of neoplastic tissues (*79*). The ratio reaches its highest value in autonomous tumors. As Furth (*2*) has pointed out, "Warburg's findings still stand as the best and perhaps only general biochemical characterization of autonomous neoplastic cells."

While the association of the Warburg phenomenon with neoplasia has never been questioned, there are differences of opinion both with respect to the chemical basis of the observed increase in glycolysis (*79, 82*) and the question of whether it represents the underlying cause or is simply a metabolic accompaniment of neoplastic transformation (*2, 39*). From the beginning Warburg has visualized the cause of all cancers as being due, specifically, to a damaged oxidative metabolism. With the development of cell-fractionation techniques and the identification of the respiratory enzymes with the mitochondria, it has been possible to localize the site of the damaged oxidative metabolism in these intracellular organelles. In his views expressed in 1956, Warburg (*79*) follows the lead of Woods and du Buy (*85, 86*) in considering mitochondria as self-replicating cytoplasmic elements which carry on an extranuclear hereditary transmission of the respiratory enzymes. The idea is also embraced that the mitochondrial substance itself can undergo mutation, or irreversible damage, which is henceforth perpetuated in descendent cells in the same way as mutated or damaged genes are perpetuated through the nucleus. The further development of neoplasia is visualized by Warburg as resulting from a selective survival of cells having the altered mitochondria. The "driving force" of the increase in fermentation, and therefore in malignancy, is said to be "the energy deficiency under which the cells operate

after destruction of their respiration, which forces the cells to replace the irretrievably lost respiration energy in some way" (*79*).

Thus, in projecting his theory involving a chemical mechanism to the biological level, Warburg has joined forces with the proponents of a burgeoning biological theory of cancer which parallels the virus theory, but in which the "continuing" causative agents are considered to be altered normal self-replicating constituents of the cytoplasm [*e.g.*, mitochondria and "plasmagenes" (*85–89*)] rather than self-replicating biological entities of extraneous origin, *i.e.*, viruses. Warburg has merely designated a specific metabolic alteration as the one and only biochemical mechanism through which such entities can act as continuing causes of cancer.

In keeping with the interpretation of dependent neoplasia as a nonspecific cellular reaction, there would be no reason to exclude altered normal self-replicating cytoplasmic components as possible continuing sources of irritating agents, *e.g.*, either abnormal products or abnormally high concentrations of normal products. However, evidence for their actual participation in neoplastic reactions in animals has not yet appeared. Nor would it be consistent with the general concept of dependent neoplasia as a reaction-to-injury to restrict the source of irritating agent to a single metabolic disorder, as proposed by Warburg. Finally, as indicated by Furth (*2*) in his "thirteen questions" regarding Warburg's concept, it would be difficult to account for all of the known types of dependent neoplasia on a basis of this single biochemical concept.

Autonomous neoplasms, however, are a different matter. They converge toward a single metabolic and dynamic biologic type, regardless of the type of continuing mechanism that initiates them and drives them during their dependent stage. It is widely believed that fermentative metabolism is a more primitive device for the production of energy than oxidative metabolism, or as Haddow (*90*) has stated it, it is "a more probable state . . . with minimal requirements for synthesis." Conversely, oxidative metabolism is a "less probable state" which might be compared with the higher degrees of morphological and functional differentiation in specialized cells of higher organisms. The loss by mitochondria of the "more differentiated" oxidative metabolism, during prolonged proliferative activity of cells, would therefore be consistent with the losses in differentiation in other respects, which characterize the phenomenon of progression in neoplasia. It is possible, therefore, that the "irretrievable loss" of oxidative metabolic machinery by cells could represent the "fundamentally different" state which distinguishes autonomous from dependent neoplasia. However, unless it can be shown that very strong concentrations of the Rous sarcoma virus act directly and specifically, within about 48 to 72 hours, to destroy the oxidative metabolic machinery of the mitochondria, it would have to be concluded that more than one mechanism can lead to the same apparent end-result, namely, that of autonomous neoplasia (*see* page 238).

Deletion Theory

The deletion theory of carcinogenesis involves a concept which long has been considered as almost axiomatic in explaining the loss in both morphological and functional differentiation sustained by the neoplastic cells of specialized tissues as they progress in degree of malignancy and anaplasia. The concept postulates the irretrievable loss of intracellular factors having to do with the related phenomena of histological structure and specialized functional activities, as the reproduction of these elements lags behind the over-all growth and reproduction of the more basic cellular constitutents during the accelerated proliferation of neoplastic cells. In fact, this same mechanism is implicit in Greenstein's (*1*) concept, now generally referred to as "the Greenstein hypothesis," of the "biochemical uniformity of tumors." Greenstein's concept was based primarily on studies of enzyme systems in neoplastic as compared with normal tissues. In spite of wide differences in enzyme patterns among tissues of different, functional, normal organs, the enzyme patterns of the neoplastic tissues deriving from such diverse organs converged toward a common, uniform, metabolic type (*1*).

The suggestion that the phenomenon of deletion might represent the underlying factor responsible for *activation* of the neoplastic reaction is a relatively new development. The experimental observations leading to this interpretation were those of Miller and Miller (*91*, *92*), which demonstrated that the carcinogenic amino azo dyes form firm complexes with normally occurring proteins of the liver, but that no similar binding takes place in the hepatic neoplasms produced by these dyes (*83*). Furthermore, the carcinogenic activity of the different compounds tested by Miller and Miller was found to be correlated with the amount or rate of binding with liver-cell proteins. Similar findings involving other carcinogenic agents as well as other tissues have been reported by Miller (*93*) and Wiest and Heidelberger (*94–96*).

The basic idea behind this theory of carcinogenesis is therefore that of an immediate deletion of certain protein constituents through direct binding with carcinogenic chemicals. Thus removed from the intracellular interacting system, they fail to undergo replication in subsequent cell divisions and are therefore irretrievably lost to all descendent cells. It is the absence of these components which is thought to deprive the affected cells of the mechanisms through which they normally respond to restraining and controlling influences of the complex organism as a whole. Lacking this restraint, the affected cells grow without host control and therefore behave in the classical manner of neoplastic cells.

A general analytical discussion of the deletion theory of carcinogenesis was presented by Rusch (*83*) in 1954. More recently Potter (*84*) has analyzed it from the viewpoint of specific enzyme systems which may suffer deletion and alter feed-back mechanisms that are concerned in the normal control of growth processes. It is significant that Potter does not implicate the deletion of any one enzyme system, or metabolic pathway,

as being the sole factor which can activate the neoplastic reaction. Nor does he confine the phenomenon of deletion to enzyme systems located in particular cytological areas or structures, such as the nucleus, cytoplasm, mitochondria, etc. On the contrary, all replicating intracellular entities are said to be subject to deletion, but it is the loss of RNA and DNA "templates" that is conceived of as being primarily responsible for the "persistent hyperplasia of cancer" (*84*). Whether similar templates might be involved in the transmission of respiratory enzymes by mitochondria was not dealt with by Warburg, but it is clear that the Warburg theory (*see* preceding section) is essentially a special case of the general deletion theory as developed by Potter. The same is true for the immunochemical hypothesis, proposed by Green (*97*) and embraced by Burnet (*98*), in which the cause of cancer is visualized as residing in the loss of certain specific antigens, called "self-markers."

The general deletion theory would also embrace Boveri's concept of the loss of chromosomal elements during multipolar mitosis (*see* page 234) since, as pointed out by Potter, the enzyme-forming systems are controlled "from above" by the DNA pattern, as well as "from below" by the molecules in the metabolic pool. The somatic mutation version of chromosomal participation in neoplasia likewise could find expression through this biochemical mechanism, as could two of the three versions of the virus theory (*see* page 236). It would require some stretch of the imagination, however, to account for cancer on a basis of this theory according to the original virus concept in which the virus is regarded as the continuing cause of the excessive tissue reaction, and the reaction itself as an extreme virus-cell interaction. But the deletion concept is broader than the term "deletion" implies, and the same metabolic end-result which is predicted by it could be achieved also through the competitive superiority of viruses in supplying their own, extraneous, "templates" for enzyme induction; *i.e.*, to the exclusion of the still present, but nonparticipating, normal templates.

It seems apparent that the concepts of the underlying biochemical and metabolic mechanisms that have been developed by the McArdle Laboratory group,[7] may be the focusing point in the "meeting of ways" among various biological as well as biochemical theories of the nature of cancer. Perhaps a different name for their conceptual structure, which would imply that it is a more general metabolic theory, would attract more attention to this fundamental area of chemical biology (*alias* biological chemistry) in which the final understanding of the reaction of autonomous neoplasia undoubtedly lies.

GENERAL DISCUSSION

It is now widely recognized that cancer is not a single disease, but a group of diversified diseases having certain pathological manifestations

[7] Drs. James and Elizabeth Miller, W. G. Wiest, Charles Heidelberger, Harold P. Rusch, and Van R. Potter; The McArdle Laboratory, Medical School, University of Wisconsin, Madison, Wis.

and dynamic biological properties in common. During recent years the idea has taken root that neoplasia in general represents a type of tissue reaction "like inflammation." Although inflammation is used as a basis of analogy, it has never been claimed that neoplasia is actually a form of inflammation, since the leukocytic infiltration which characterizes the inflammatory reaction does not constitute a primary component of neoplasia. What, then, is the general tissue reaction with which neoplasia can be precisely compared? It has been the chief purpose of this communication to show that another well-recognized component of the overall "reaction-to-injury," namely, that involving the primary, primitive responses of individual cells to irritation, is the actual tissue-reaction involved. This component of the general reaction is separated for purposes of discussion in textbooks on pathology under the designation "repair"; but the persistence of the teleological concept that "repair" has a specific "purpose" and is therefore supposed to stop when all lost or damaged tissue has been restored[8] has delayed the clear recognition of this fundamental process as one that can continue beyond the normal limits of "repair."

As already pointed out, "repair" is accomplished through only two basic biological responses of cells to irritation, namely, *proliferation* and *migration*. Also, the prolonged stimulation of these same responses can lead to varying degrees of hyperplasia as well as to dependent neoplasia. Whether autonomous neoplasia is basically the same type of phenomenon remains to be determined, but it is clear that even in this extreme oncological reaction, the primary manifestations of the cells involved are those of proliferation and migration.

Since the term "repair" does not allow for an extension of these basic cellular responses *beyond* physiological limits, a new term is needed (as a companion of "inflammation") for embracing this additional component of the general reaction-to-injury when it progresses beyond recognized physiological limits. For the want of a better word, *cytoplasia* is tentatively employed for the purpose. Strictly speaking, this term does not include the phenomenon of migration, and the substitution of a more appropriate word would be welcomed; but as here defined, cytoplasia indicates the twin responses of proliferation and migration which go together, though not simultaneously in a given cell, in the reactions of individual cells to irritating agents in general. Under physiological conditions cytoplasia provides the functional basis for repair, but when abnormal stimuli are excessively prolonged, the continued cytoplasia results in varying degrees of hyperplasia, grading imperceptibly into dependent neoplasia.

By such identification of dependent neoplasia with these long-recognized and highly familiar cellular reactions, the dependent form of neoplastic disease loses much of its former mystery. Also, analogies, such as "outlaw," "anarchist," "juvenile delinquent," etc., which have been popularly

[8] A typical statement (37, page 61), which appeared as late as 1929, illustrates this limited point of view: "Cells proliferate only to replace cells of their own kind which have been destroyed."

used for depicting the behavior of cancer cells in lay discussions, lose their glamour. In the dependent form of the disease at least, the cells do not behave in a manner "contrary to society," but simply do what any "self-respecting" cell would do under the same circumstances, that is, react in self-defense by any means at their disposal as individuals. It is simply an unfortunate circumstance, from the viewpoint of the individual cell, that the primitive defensive reaction can be detrimental to the host-organism as a whole. [*See* Schwartz and Schoolman (*14*) for a similar interpretation with respect to leukemia.]

Whether or not autonomous neoplasia is eventually shown to belong to this same general class of tissue reactions, the mystery surrounding autonomous tumors is also lessened considerably by the knowledge that, in laboratory animals at least, most if not all autonomous tumors develop from pre-existing dependent tumors or precancerous lesions (*2*). Two types of evidence indicate that this may be true also for human neoplasms: (1) In his study of the natural history of human neoplastic disease, Shimkin (*17*) has shown that in many types of cancer the mean duration of life after the apparent clinical onset of the disease is from 1 to 3 years, even in untreated patients. In a small percentage of the cases charted by Shimkin, the duration of life in the absence of treatment was as long as 5 to 7 years. Although the lesions were not identified with respect to dependency or autonomy, it would seem safe to conclude that in those cases in which the patient survived for more than a few months, the neoplastic lesions were not entirely beyond some degree of control by their hosts. (2) The second type of evidence is that described by Harold Stewart (*16*) in his discussion of cutaneous carcinomas. Among human neoplasias, those involving the external surfaces are the only ones that can be detected under practical conditions in their earliest stages, and also correlated with pre-existing lesions at the same sites. In his analysis of neoplastic lesions of the skin, Stewart lists 14 types of precancerous lesions which may precede the malignant growths in man, and makes the following summarizing statements with respect to their origin: "Whether cutaneous cancer can ever develop in normal tissue or whether every malignant tumor is preceded by some abnormality has not been definitely established. However, the fact remains that in man carcinoma is regularly preceded by a lesion which, while it cannot be called cancer or even always precancer, from the standpoint of pathology, yet provides a favorable soil for neoplastic development." This clearly suggests that the situation in man may not be too different from that in laboratory animals, and that spontaneous autonomous skin tumors of man may be expected to have their origin in pre-existing pathological tissue reactions of some type, including dependent neoplasms.

A final subject for comment involves the implications of the foregoing interpretations with respect to the prevention and early diagnosis of cancer. If, as suggested, autonomous tumors for the most part have their origin in dependent or precancerous lesions, the prevention or control of the latter lesions would remove the basis for development of a large majority

of autonomous neoplasms. There is hope, therefore, that even this largely unknown facet of the over-all cancer problem can be controlled to a significant degree by increased knowledge of the precancerous and dependent neoplastic lesions. Considerable progress has already been made both in the diagnosis and control of dependent neoplasias having their origin in hormonal dyscrasias (*8*). However, these do not represent a very large segment of the cancer picture as a whole, and not much of a dent has been made in the over-all problem by their successful control. Similarly, great progress has been made in the detection and elimination of carcinogenic stimuli (chemicals and radiation) from the human environment, but, again, this has not greatly altered the incidence of the human disease. By far the greatest majority of spontaneously occurring human neoplasms are associated with etiological agents that are still unknown (*30*). The possibility that viruses may be involved in the human diseases to a considerable degree is suggested by the discovery of many new tumor viruses of laboratory animals during the past few years. Intensive programs in search of such agents in humans are already under way in many laboratories. Assuming that an appreciable number will be found, what will be the prospects for prevention and early diagnosis of the dependent neoplastic diseases? Judging from the experiences thus far with the known virus tumors of laboratory animals, prevention may be a practical possibility in some cases where the agent can be eliminated, *e.g.*, as in mammary-gland cancer of mice in which the etiological agent is transmitted primarily through the mother's milk (*56*), or in fowl lymphomatosis (*99*) in which the agent is transmitted, by contact, through secretory and excretory products. On the other hand, prevention may be entirely impossible in other cases, as for example, in pulmonary adenoma of mice, in which the etiological agents, conceivably viral nucleic acids, may be transmitted through the chromosomes of the sex cells, as "parasitic genes." As for early diagnosis, with the exception of the newly discovered polyoma virus of mice (*100*)—which appears to be a maverick among tumor viruses in several respects—tumor viruses of laboratory animals have been notably difficult to detect with presently available serological and immunological methods under conditions which approach those of the naturally occurring diseases. It is probable that more sensitive diagnostic methods will be devised. However, even assuming the existence of adequate methods, a single "test for cancer" cannot be hoped for. On the contrary, as with the viruses of infectious diseases the detection of viruses associated with neoplasia in humans will undoubtedly require a battery of diagnostic tests.

REFERENCES

(*1*) GREENSTEIN, J. P.: Biochemistry of Cancer, 2d ed. New York, Academic Press, Inc., 1954, 653 pp.

(*2*) FURTH, J.: A meeting of ways in cancer research: Thoughts on the evolution and nature of neoplasms. Cancer Res. 19: 241–258, 1959.

(3) FURTH, J.: Conditioned and autonomous neoplasms. A review. Cancer Res. 13: 477–492, 1953.
(4) FURTH, J., and METCALF, D.: An appraisal of tumor-virus problems. J. Chronic Dis. 8: 88–112, 1958.
(5) STRONG, L. C.: The induction of mutations by a carcinogen. Brit. J. Cancer 3: 97–108, 1949.
(6) GARDNER, W. U.: Hormonal imbalances in tumorigenesis. Cancer Res. 8: 397–411, 1948.
(7) ———: Hormones and carcinogenesis. Canad. Cancer Conf. 2: 207–241, 1956.
(8) HUGGINS, C.: Control of cancers of man by endocrinologic methods. A review. Cancer Res. 16: 825–830, 1956.
(9) HERTZ, R.: An appraisal of the concepts of endocrine influence on the etiology, pathogenesis, and control of abnormal and neoplastic growth. Cancer Res. 17: 423–431, 1957.
(10) FOULDS, L.: The experimental study of tumor progression. A review. Cancer Res. 14: 327–339, 1954.
(11) ———: The histologic analysis of mammary tumors of mice. J. Nat. Cancer Inst. 17: 701–801, 1956.
(12) GREENE, H. S. N.: A conception of tumor autonomy based on transplantation studies. A review. Cancer Res. 11: 899–903, 1951.
(13) ———: The significance of the heterologous transplantability of human cancer. Cancer 5: 24–44, 1952.
(14) SCHWARTZ, S. O., and SCHOOLMAN, H. M.: The etiology of leukemia: The status of the virus as causative agent—a review. Blood 14: 279–294, 1959.
(15) DURAN-REYNALS, F.: Virus-induced tumors and the virus theory of cancer. *In* The Physiopathology of Cancer (Homburger, F., ed.). New York, Paul B. Hoeber, Inc., Harper & Brothers, 1959, pp. 238–292.
(16) STEWART, H. L.: Experimental cutaneous carcinomas. *Ibid.* 1959, pp. 3–17.
(17) SHIMKIN, M. B.: Natural history of neoplastic disease. *Ibid.* 1959, pp. 855–871.
(18) DUNN, T. B.: Morphology of mammary tumors in mice. *Ibid.* 1959, pp. 38–84.
(19) PETROV, N. N.: A new definition of the conception of tumor growth. *In* Pathogenesis and Immunology of Tumors (Vygodchikov, C. V., ed.). English translation by R. Crawford, New York, Pergamon Press, 1959.
(20) KIT, S., and GRIFFIN, A. C.: Cellular metabolism and cancer. A review. Cancer Res. 18: 621–656, 1958.
(21) BARRETT, M. K.: A critical analysis of tumor immunity. J. Chron. Dis. 8: 136–157, 1958.
(22) ROUS, P.: The nearer causes of cancer. J.A.M.A. 122: 573–581, 1943.
(23) BEARD, J. W.: Viruses as a cause of cancer. Am. Scientist 46: 226–254,1958.
(24) STANLEY, W. M.: The virus etiology of cancer. *In* Proc. Third National Cancer Conf. Philadelphia, J. B. Lippincott Co., 1957, pp. 42–51.
(25) ANDERVONT, H. B.: Problems concerning the tumor viruses. *In* The Viruses (Burnet, F. M., and Stanley, W. M., eds.). New York, Academic Press, Inc., 1959, pp. 307–368.
(26) HIRSCHBERG, E.: A list of review articles on cancer research 1951–1955. Cancer Res. 17: 77–85, 1957.
(27) EWING, J.: Neoplastic Diseases. Philadelphia, W. B. Saunders Co., 1931.
(28) BRYAN, W. R.: Host virus relationships in tumor inducing viruses. Texas Rep. Biol. & Med. 15: 674–703, 1957.
(29) GOODPASTURE, E. W.: The pathology of viral neoplasia. Texas Rep. Biol. & Med. 15: 451–461, 1957.
(30) BOYD, W.: A Text-Book of Pathology: An Introduction to Medicine, 6th ed. Philadelphia, Lea & Febiger, 1953, 1024 pp.
(31) MCCUTCHEON, M.: Inflammation. *In* Pathology (Anderson, W. A. D., ed.), 2d ed. St. Louis, C. V. Mosby Co., 1953, pp. 13–62.

(*32*) METCHNIKOFF, E.: Lectures on the Comparative Pathology of Inflammation. English transl. by F. A. and E. H. Starling. London, 1893. [Quoted by McCutcheon (*31*).]

(*33*) PINKERTON, H.: Rickettsial and viral diseases. *In* Pathology (Anderson, W. A. D., ed.), 2d ed. St. Louis, C. V. Mosby Co., 1953, pp. 299–339.

(*34*) FORBUS, W. D.: Reaction to Injury: Pathology for Students of Disease Based on the Functional and Morphological Responses of Tissues to Injurious Agents. Baltimore, Williams & Wilkins Co., 1943, 797 pp.

(*35*) MACCALLUM, W. C.: A Text-Book of Pathology, 5th ed. Philadelphia, W. B. Saunders Co., 1930, 1212 pp.

(*36*) WILLIS, R. A.: The Pathology of Tumors. St. Louis, C. V. Mosby Co., 1948.

(*37*) MALLORY, F. B.: The Principles of Pathologic Histology. Philadelphia, W. B. Saunders Co., 1929.

(*38*) FISCHER, B.: Die experimentelle Erzeugung atypischer Epithel-wucherungen und die Entstehung bösartiger Geschwülste. München med. Wchnschr. 53: 2041, 1906. [Quoted by Rous (*22*).]

(*39*) FARDON, J. C.: A reconsideration of the somatic mutation theory of cancer in the light of some recent developments. Science 117: 441–445, 1953.

(*40*) BOVERI, T.: The Origin of Malignant Tumors. English translation by M. Boveri. Baltimore, Williams & Wilkins Co., 1929.

(*41*) Fundamental Cancer Research: Report of a Committee Appointed by the Surgeon General. Pub. Health Rep. 53: 2121–2130, 1938. Reprinted in J. Nat. Cancer Inst. 19: 317–325, 1957.

(*42*) ROUS, P., and ROGERS, S.: The occurrence in tarred rabbit skin of minor, almost imperceptible, neoplastic changes. Cancer Res. 12: 292–293, 1952.

(*43*) BERENBLUM, I.: Carcinogenesis and tumor pathogenesis. Advances Cancer Res. 2: 129–175, 1954.

(*44*) ROUS, P., and KIDD, J. G.: Conditional neoplasms and subthreshold neoplastic states, a study of the tar tumors of rabbits. J. Exper. Med. 73: 365–390, 1941.

(*45*) BLUM, H. F.: On the mechanism of cancer induction by ultraviolet radiation. J. Nat. Cancer Inst. 11: 463–495, 1950.

(*46*) ———: Carcinogenesis by Ultraviolet Light. Princeton, N.J., Princeton Univ. Press, 1959.

(*47*) BORREL, A.: Epithelioses infectieuses et epitheliomas. Ann. Inst. Pasteur 17: 81–122, 1903.

(*48*) OBERLING, C.: The Riddle of Cancer, rev. ed. English translation by W. H. Woglom. New Haven, Yale Univ. Press, 1952, 238 pp.

(*49*) ROUS, P.: The virus tumors and the tumor problem. Am. J. Cancer 28: 233–272, 1936.

(*50*) ANDREWES, C. H.: Latent virus infections and their possible relevance to the cancer problem: president's address. Proc. Roy. Soc. Med. 33: 75–86, 1939.

(*51*) OBERLING, C., and GUÉRIN, M.: The role of viruses in the production of cancer. Advances Cancer Res. 2: 353–423, 1954.

(*52*) KIDD, J. G.: Proliferative lesions caused by viruses and virus-like agents. *In* The Pathogenesis and Pathology of Viral Diseases (Kidd, J. G., ed.). New York, Columbia Univ. Press, 1950, pp. 161–180.

(*53*) SYVERTON, J. T.: The pathogenesis of the rabbit papilloma-to-carcinoma sequence. Ann. New York Acad. Sc. 54: 1126–1140, 1952.

(*54*) LWOFF, A., SIMINOVITCH, L., and KJELGAARD, N.: Induction de production de phage dans une bacterie lysogine. Ann. Inst. Pasteur 78: 815–858, 1950.

(*55*) LURIA, S. E.: General Virology. New York, John Wiley & Sons, Inc., 1953.

(*56*) BITTNER, J. J.: Some possible effects of nursing on the mammary gland tumor incidence in mice. Science 84: 162, 1936.

(*57*) Staff, Jackson Memorial Laboratory: The existence of non-chromosomal influence in the incidence of mammary tumors in mice. Science 78: 465–466, 1933.

(58) Law, L. W.: Genetic studies in experimental cancer. Advances Cancer Res. 2: 281–352, 1954.

(59) Gross, L.: Filterable agent causing leukemia following inoculation into newborn mice. Texas Rep. Biol. & Med. 15: 603–626, 1957.

(60) Heston, W. E.: Effects of genes located on chromosomes, III, V, VII, IX, and XIV on the occurrence of pulmonary tumors in the mouse. *In* Proc. Internat. Genetics Symp., 1956. Supp. Vol. Cytologia 219–224, 1957.

(61) Epstein, M. A., and Holt, S. J.: Observations on the Rous virus: integrated electron-microscopical and cytochemical studies of fluorocarbon purified preparations. Brit. J. Cancer 12: 363–369, 1958.

(62) Epstein, M. A.: Observations on purified preparations of the Rous virus: infectivity and structure of the particles. Acta Unio internat. contra cancrum 15: 814–818, 1959.

(63) Bernhard, W., Oberling, C., and Vigier, P.: Ultrastructure de virus dans le sarcome de Rous, leur rapport avec le cytoplasme des cellules tumorales. Bull. du Cancer 43: 407–422, 1956.

(64) Malmgren, R. A., Fink, M. A., and Mills, W.: Demonstration of the intracellular location of Rous sarcoma virus by fluorescein-labeled antisera. J. Nat. Cancer Inst. In press, April 1960.

(65) Rubin, H., and Temin, H. M.: Infection with the Rous sarcoma virus in vitro. Fed. Proc. 17: 994–1003, 1958.

(66) Loomis, L. N., and Pratt, A. W.: The histogenesis of Rous sarcoma I induced by partially purified virus. J. Nat. Cancer Inst. 17: 101–121, 1956.

(67) Beard, J. W.: The chemical, physical and morphological properties of animal viruses. Physiol. Rev. 28: 349–367, 1948.

(68) Noyes, W. F., and Mellors, R. C.: Flourescent antibody detection of the antigens of the Shope papilloma virus in papillomas of the wild and domestic rabbit. J. Exper. Med. 106: 555–562, 1957.

(69) Palmer, C. G.: The cytology of rabbit papillomas and derived carcinomas. J. Nat. Cancer Inst. 23: 241–249, 1959.

(70) Rous, P., Kidd, J. G., and Beard, J. W.: Observations on the relation of the virus causing rabbit papillomas to the cancers deriving therefrom. I. The influence of the host species and of the pathogenic activity and concentration of the virus. J. Exper. Med. 64: 385–400, 1936.

(71) Rous, P., and Friedewald, W. F.: The carcinogenic effect of methylcholanthrene and of tar on rabbit papillomas due to a virus. Science 94: 495–496, 1941.

(72) Rous, P., and Kidd, J. G.: The carcinogenic effect of a papilloma virus on the tarred skin of rabbits. I. Description of the phenomenon. J. Exper. Med. 67: 399–428, 1938.

(73) Rogers, S., and Rous, P.: Joint action of a chemical carcinogen and a neoplastic virus to induce cancer in rabbits; results of exposing epidermal cells to a carcinogenic hydrocarbon at time of infection with the Shope papilloma virus. J. Exper. Med. 93: 459–488, 1951.

(74) Noyes, W. F.: Studies on the Shope rabbit papilloma virus. II. The location of infective virus in papillomas of the cottontail rabbit. J. Exper. Med. 109: 423–428, 1959.

(75) Shope, R. E.: Immunization of rabbits to infectious papillomatosis. J. Exper. Med. 65: 219–231, 1937.

(76) Fraenkel-Conrat, H.: The infectivity of tobacco mosaic virus nucleic acid. *In* Cellular Biology, Nucleic Acids and Viruses (St. Whitlock, O. V., ed.). New York, New York Acad. Sc., 1957, p. 217.

(77) Holland, J. J., McLaren, L. C., and Syverton, J. T.: The mammalian cell virus relationship. IV. Infection of naturally insusceptible cells with enterovirus ribonucleic acid. J. Exper. Med. 110: 65–80, 1959.

(78) Warburg, O., ed.: The Metabolism of Tumours. English transl. by F. Dickens. London, Constable & Co., Ltd., 1930, 327 pp.

(79) ———: On the origin of cancer cells. Science 123: 309–314, 1956.
(80) ———: On respiratory impairment in cancer cells. Science 124: 269–270, 1956.
(81) WEINHOUSE, S., WARBURG, O., BURK, D., and SCHADE, A. L.: On respiratory impairment in cancer cells. Science 124: 267–272, 1956.
(82) BURK, D., and SCHADE, A. L.: On respiratory impairment in cancer cells. Science 124: 270–272, 1956.
(83) RUSCH, H. P.: Carcinogenesis: A facet of living processes. Cancer Res. 14: 407–417, 1954.
(84) POTTER, V. R.: The biochemical approach to the cancer problem. Fed. Proc. 17: 691–697, 1958.
(85) WOODS, M. W., and DUBUY, H. G.: Cytoplasmic diseases and cancer. Science 102: 591–593, 1945.
(86) DUBUY, H. G., and WOODS, M. W.: A possible common mitochondrial origin of the variegation and virus diseases in plants and cancers in animals. *In* Am. Assoc. Adv. Sc. Research Conference on Cancer. Washington, D.C., Am. Assoc. Adv. Sc., 1945, pp. 162–169.
(87) DARLINGTON, C. D.: Heredity, development and infection. Nature, London 154: 164–169, 1944.
(88) ———: Paracrinkle virus and inheritance. Nature, London 154: 489, 1944.
(89) HADDOW, A.: Transformation of cells and viruses. Nature, London 154: 194–199, 1944.
(90) ———: The biochemistry of cancer. Ann. Rev. Biochem. 24: 689–742, 1955.
(91) MILLER, E. C., and MILLER, J. A.: Symposium on immunogenetics and carcinogenesis; in vivo combinations between carcinogens and tissue constituents and their possible role in carcinogenesis. Cancer Res. 12: 547–556, 1952.
(92) MILLER, J. A., and MILLER, E. C.: The carcinogenic aminoazo dyes. Advances Cancer Res. 1: 339–396, 1953.
(93) MILLER, E. C.: Studies on the formation of protein-bound derivatives of 3,4-benzpyrene in the epidermal fraction of mouse skin. Cancer Res. 11: 100–108, 1951.
(94) WIEST, W. G., and HEIDELBERGER, C.: The interaction of carcinogenic hydrocarbons with tissue constituents. I. Methods. Cancer Res. 13: 246–249, 1953.
(95) ———: The interaction of carcinogenic hydrocarbons with tissue constituents. II. 1,2,5,6-dibenzanthracene-9,10-C^{14} in skin. Cancer Res. 13: 250–254, 1953.
(96) ———: The interaction of carcinogenic hydrocarbons with tissue constituents. III. 1,2,5,6-dibenzanthracene-9,10-C^{14} in the submaxillary gland. Cancer Res. 13: 255–261, 1953.
(97) GREEN, H. N.: An immunological concept of cancer: a preliminary report. Brit. M. J. 2: 1374–1380, 1954.
(98) BURNET, F. M.: Enzyme antigen and virus. Cambridge, Cambridge Univ. Press, 1956.
(99) BURMESTER, B. R., and GENTRY, R. F.: The transmission of avian visceral lymphomatosis by contact. Cancer Res. 14: 34–42, 1954.
(100) STEWART, S. E., and EDDY, B. E.: Properties of a tumor-inducing virus recovered from mouse neoplasms. *In* Perspectives in Virology (Pollard, M., ed.). New York, John Wiley & Sons, Inc., 1959.

Announcement

Manuscripts Approved for JNCI, November 15 to December 15, 1959

"Studies on eight transplantable plasma-cell neoplasms of mice," by Michael Potter and John L. Fahey.

"Statistics on men disqualified for military service because of cancer (a note)," by Bernard D. Karpinos.

"Induction of tumors in Syrian hamsters by a cytopathogenic virus derived from a C3H mouse mammary tumor," by A. A. Axelrad, E. A. McCulloch, A. F. Howatson, A. W. Ham, and L. Siminovitch.

"The histopathological sequence in viral carcinogenesis in the hamster kidney," by A. W. Ham, E. A. McCulloch, A. A. Axelrad, L. Siminovitch, and A. F. Howatson.

"*In vitro, in vivo,* and electron microscope studies of a virus recovered from a C3H mouse mammary tumor: Relationship to polyoma virus," by A. F. Howatson, E. A. McCulloch, J. D. Almeida, L. Siminovitch, A. A. Axelrad, and A. W. Ham.

"Estrogen-induced kidney tumors in the golden hamster. I. Biochemical composition during tumorigenesis," by Robert F. McGregor, Joseph D. Putch, and Darrell N. Ward.

"The DNA content of tumor cells. II. Alterations during the formation of hepatomas in rats," by H. F. Stich.

"A chromatographic study of the deoxyribonucleic acids from normal and leukemic human tissues," by Giampiero di Mayorca, Herbert S. Rosenkranz, Elio E. Polli, Grace C. Korngold, and Aaron Bendich.

"The growth of estrogen-induced interstitial cell testicular tumors in BALB/c mice," by H. B. Andervont, M. B. Shimkin, and H. Y. Canter.

"An evaluation of the proposed international clinical staging system for cancer of the breast," by Calvin Zippin and Henry I. Kohn.

"Postcoupling, noncoupling, and fluorescence techniques for the demonstration of alkaline phosphatase," by M. S. Burstone.

"Acceptance of rat and mouse lung grafts by radiation chimeras," by George W. Santos, Richard M. Garver, and Leonard J. Cole.